INFLUENCE OF IONS ON THE INTERACTION OF INFLUENZA VIRUS AND CELLULAR RECEPTORS OR SOLUBLE INHIBITORS OF HAEMAGGLUTINATION

by F. M. BURNET[1] AND MARGARET EDNEY[2]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

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It has long been known that in the absence of ions many specific biological reactions fail to occur. Examples are readily found in the fields of antigen-antibody reaction, enzyme-substrate interaction and adsorption of bacterial viruses to the host cell. Recently Lowell and Buckingham (1948) and Davenport and Horsfall (1948) found by the use of red cells suspended in isotonic glucose that influenza virus was likewise not adsorbed to the cell surface in the absence of ions. It has probably been usual to regard the necessity of ions in the system merely as one of the necessary background elements such as temperature, pH, viscosity, against which the reaction took place. There is, however, an increasing tendency to look for a more specific function of ions particularly in relation to enzyme action (review by Lehninger, 1950) and a recent paper by Puck, Garen and Cline (1951) makes a strong case for the specificity of salt action in adsorption of bacterial viruses to the host cell surface. This work on bacterial viruses made it highly desirable to examine the rôle of ions in relation to influenza virus reactions both with the red cell surface and with soluble mucoid inhibitors.

In the present series of three papers the results are presented of an experimental examination of the rôle of ions in most of the *in vitro* reactions of influenza viruses which have been studied in this laboratory. The present paper is concerned with (a) the conditions for macroscopic haemagglutination, (b) adsorption of virus to red cells in the cold, (c) red cell receptor destruction by viruses, (d) the union of indicator virus with mucoid and (e) the enzymic action of virus on a typical mucoid inhibitor. For all these actions ions are necessary though the specific requirements may differ from one reaction to another. In the second paper (Burnet, 1952) an incidental finding, the occurrence of irreversible union of indicator virus to the red cell, is elaborated. The third paper (Edney and Burnet, 1952) is concerned with the conditions for inactivation of haemagglutinin and production of indicator virus.

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[2] Francis Haley Research Officer, Department of Experimental Medicine, University of Melbourne.

Materials and Methods.

Virus. Two virus strains only have been studied in detail, MEL A and LEE B. These are well known influenza strains and have been used in the form of allantoic fluid from chick embryos inoculated at 11 days' incubation with a high dilution of seed virus and harvested after 44 hours' incubation at 35°C. The fluids were dialysed for 48 hours through sacs made of "Visking" sausage casing, against 0·001 M borate buffer (Clark, 1920) at 4° C. The final pH of the dialysed virus was 7·2 to 7·4 and the haemagglutinin titre, by the pattern method used in this laboratory, between 500 and 1,200.

When indicator virus was needed this was prepared in the case of LEE by simply heating the dialysed virus to 56°C. for 30 minutes. With MEL virus "Calgon" (sodium hexametaphosphate) was added to give a concentration of 0·0002 M before heating. This was sufficient to allow change to indicator without having any significant effect on the subsequent experiments with the virus. The indicators are referred to as H.LEE and T.MEL.

Fowl erythrocytes were used in all experiments except for the preparation of "PVR cells" mentioned later. We were unable to prepare stable suspensions of fowl cells in pure glucose solutions and the glucose solution used contained 0·0025 M sodium phosphate. With this diluent no difficulty was encountered in handling the cells.

Buffers. A series of sodium phosphate solutions were kindly prepared by Mr. C. C. Curtain by mixing appropriate amounts of 0·10M NaH_2PO_4 and 0·10M NaOH to give buffers of pH 6·0, 7·0, 8·0, all of which contained equivalent 0·10M Na^+ concentrations.

Citrate saline 2 p.c. sodium citrate in normal saline.

Calcium acetate saline (*CA saline*) the standard medium for RDE action contains per litre — Sodium acetate 12·3 gm. NaCl 5·0 gm. $CaCl_2$ 1·0 with acetic acid (approximately 1·3 ml. 2N) to bring pH to 6·2.

Salt solutions. Molar solutions of A.R. salts in distilled water were used as stock solutions and further dilutions prepared as required.

Receptor-destroying Enzyme. (*RDE*). The purified enzyme was kindly provided by Mr. G. L. Ada in the form of a solution in CA saline containing 8,000 units per 0·25 ml. It was prepared by the method of Ada and French (1950) and titrated according to Burnet and Stone (1947). The RDE solution was distributed in small tubes and stored frozen at —10° C.

Meconium inhibitor. The preparation of semi-purified meconium inhibitor will be described by French, Curtain and Pye in a forthcoming paper. Its general properties are closely similar to those of the ovarian cyst mucoid described by McCrea (1948). It is extremely stable and can be boiled without loss of inhibitor titre as well as being fully soluble in distilled water and salt solutions of the range used in the present work. The stock solution used was a dialysed preparation containing approximately 0·4 p.c. of solid.

PVR cells. These were prepared essentially by the method of Fazekas de St.Groth (1949). Washed human cells were used, initially because of a temporary shortage of fowl cells but as they proved satisfactory their use was continued. In a total volume of 5 ml. made up with normal saline, 0·5 ml. of packed human cells and 0·5 ml. of 0·0013 M KIO_4 were allowed to react for 15 minutes at room temperature. The cells were then spun down and the supernatant discarded. The cells were now resuspended in 5 ml. of virus appropriately converted to the indicator state. Rapid complete agglutination occurred and after 10 minutes the cells were spun down and the supernatant replaced by 5 ml. of CA saline containing 0·25 ml. of RDE (8,000 units). The cells were stabilized by one hour's exposure to 37° C. in a water-bath, washed with 2 p.c. citrate saline and made up to a stock 20 p.c. suspension in the same medium. The 1 p.c. suspension used in inhibitor titrations was prepared by diluting this in normal saline.

The use of such cells to which indicator virus was attached for titrations of inhibitor was referred to by Fazekas de St.Groth (1949) but it has not previously been used as a general

experimental method. Titrations have been made by preparing serial dilutions of inhibitor in saline and adding an equal volume of 1 p.c. PVR cells. A modification which has proved convenient is to make the inhibitor dilutions in 0·5 p.c. suspension of treated cells. Owing to the wide range of titre which may be involved in, for example, titrations of enzymic action we have used a factor of 3·16 ($\sqrt{10}$) between tubes, i.e. 0·54 ml. of cell suspension in each tube with transfer of 0·25 ml. from one tube to the next in series.

Except for minor variations referred to in the text the four standard types of experiment were carried out as follows:

Requirements for haemagglutination: Serial dilutions of salt were made in 5 p.c. glucose solution, an equal volume (0·25 ml.) of virus diluted in glucose solution to 10 standard agglutinating doses and one drop of 20 p.c. cells in phosphate glucose were added to each tube and the pattern read when the cells had settled at room temperature.

Absorption of virus: Using chilled reagents 0·25 ml. of 20 p.c. fowl cells in glucose solution was added to a mixture containing 0·25 ml. of undiluted virus and the required salt concentration, so that in a total volume of 1·25 ml. virus dilution was 1 : 5, glucose 5 p.c., cells 4 p.c. by volume and in addition to the salt added for the experiment, small amounts not equivalent to more than 0·001 M from the reagents.

After 1 hour at 0–4° C. the tubes were spun and the supernatant fluid removed for titration. The cells were resuspended in 1 ml. of CA saline and 1 drop of RDE solution (titre, 8,000) added. The tubes were held in a waterbath at 37° C. for 30 minutes to allow liberation of the virus and then spun. The supernatant fluid was titrated in citrate saline to allow estimation of haemagglutinin in the presence of active RDE.

Enzymic action: The standard conditions used involved the exposure of 1 : 50 dilution of a standard solution of semi-purified meconium inhibitor to 10 agglutinating doses of virus for 2 hours at 37° C. The dialysed virus was diluted in distilled water to a concentration of 50 agglutinating doses and an appropriate series of salt dilutions made in distilled water so that the concentrations were 5 times those needed in the final mixture. The third reagent was a 1 : 10 dilution in distilled water of semi-purified and dialysed meconium inhibitor. In each experimental mixture 0–25 ml. of each reagent was present with the volume made up to 1·25 ml. with distilled water. The appropriate controls, inhibitor alone and inhibitor plus virus without salt, were always included. The pH of the mixtures was always within the range 7·0–7·4, the small amount of borate in the dialysed virus acting as a buffer. The reaction of each tube was checked at the conclusion of the experiments by addition of a drop of universal indicator.

After 2 hours' incubation the tubes were placed in a boiling water bath for 1 minute to destroy haemagglutinin and the residual inhibitor titrated against 5 agglutinating doses of the indicator viruses H.LEE and T.MEL by the standard method used in this laboratory (Burnet, 1948). In some of the experiments to be reported duplicate titrations of inhibitor were made using Fazekas de St.Groth's method in which "PVR cells" prepared with H.LEE and T.MEL are directly agglutinated.

EXPERIMENTAL.

Influence of salts on macroscopic haemagglutination by viruses.

In these experiments serial dilutions of the salts required were made in 5 p.c. glucose solution, the cells were prepared as described and suspended in 5 p.c. glucose containing phosphate 0·0025 M in terms of Na^+. Dialysed virus was diluted with glucose solution so that one drop (0·025 ·ml.) added to each

tube would give a concentration equivalent to 10 agglutinating doses. Na, K, Ca and Mg chlorides were studied and a variety of other sodium salts. It was found that cells in glucose were highly susceptible to agglutination by Ca salts in the absence of virus with a rather irregular end-point. In some experiments virus agglutination occurred slightly beyond the end-point of spontaneous Ca agglutination which varied between 0·005 M and 0·002 M with different batches of cells.

Tables 1 and 2 show the lowest concentration of salts in which the virus gives standard one-plus partial agglutination.

TABLE 1.

End-point of haemagglutination with different chlorides.

Virus	Molarity			
	NaCl	KCl	$CaCl_2$	$MgCl_2$
MEL	0·014	0·0083	—	0·0067
T. MEL	0·005	0·0025	0·001	0·0018
LEE	0·013	0·0078	—	0·0042
H. LEE	0·005	0·005	—	0·0031

— Agglutination not significantly higher than with Ca alone (0·002 M). The figures in Tables 1 and 2 show the lowest concentration of salt capable of giving agglutination with 10 agglutinating doses of the viruses shown.

TABLE 2.

End-point of haemagglutination by LEE with different salts.

	Salt	Concentration
	Sodium chloride	0·0091 N
	„ nitrate	0·0083 N
	„ sulphate	0·0083 N
	„ oxalate	0·0083 N
	„ citrate	0·0107 N
*	„ hexametaphosphate	0·0094 N

* Calgon has been assumed to have the formula $(NaPO_3)_6$.

It will be seen that K is slightly more active than Na and the divalent cations, as far as they can be studied, more active than the monovalent ones. Indicator viruses show agglutination to a lower salt concentration than active viruses.

Absorption of virus by cells in relation to ionic status of medium.

Under the experimental conditions used effective absorption of active virus rarely occurred at concentrations below 0·10 M of salt but there was no evidence of any sharp end-point. It was unusual to obtain complete absence of any apparent absorption in the mixtures without added salt and there was always a small, sometimes a moderate, amount eluted from the corre-

sponding cells by RDE. As with straight haemagglutination tests absorption of indicator virus was effective at a lower salt concentration than active virus.

Figs. 1, 2 and 3 give representative results.

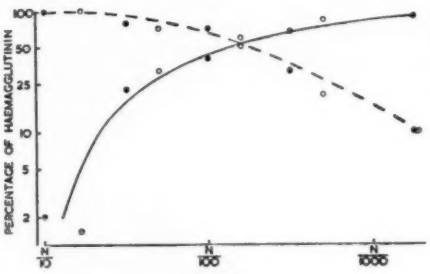

Fig. 1.

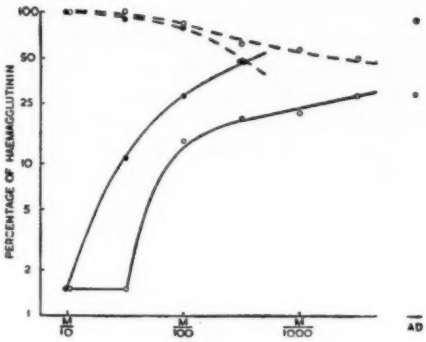

Fig. 2.

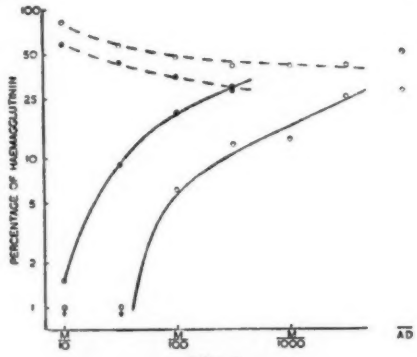

Fig. 3.

Fig. 1. Absorption and elution by RDE of LEE virus to fowl erythrocytes in varying salt concentrations.

In this Figure, and in Figs. 2 and 3, the ordinates represent percentage haemagglutinin remaining after absorption (continuous line) or released by subsequent treatment with RDE (broken line). Abcissae indicate the salt concentration.

● NaCl ○ Na_2SO_4.

Fig. 2. Absorption and elution of virus LEE in NaCl and $CaCl_2$ solutions.

● NaCl ○ $CaCl_2$.

AD indicates that no additional salt was added beyond that unavoidably present in the reagents.

Fig. 3. Absorption and elution of indicator virus H. Lee in NaCl and $CaCl_2$ solutions.

● NaCl ○ $CaCl_2$.

In Fig. 1 is a comparison of NaCl and Na_2SO_4 to indicate the concordance of curves of absorption and RDE elution when the cation concentrations are equivalent. Figs. 2 and 3 present the results of tests with LEE in the active

and indicator state using NaCl and $CaCl_2$ dilutions. Results have always conformed to these general patterns but it has been found impossible to obtain strictly reproducible results when different batches of viruses and red cells have necessarily to be used. In some of the earlier experiments in which every effort was made to have the lowest possible concentration of salts in the control mixture it was found that a relatively large amount of virus was absorbed but not released after treatment with RDE. This was not shown when 0·001 M phosphate was included deliberately and for this reason in all the experiments shown in the Figures the control contains sodium phosphate and traces of other salts equivalent to 0·001 M Na.

Behaviour of red cells "modified" by periodate in regard to ions.

In view of the possibility that cells modified by periodate treatment might react in a different fashion from normal cells experiments which indicate their essential similarity of behaviour to that of normal cells will be briefly reported.

TABLE 3.

Failure of periodated cells to adsorb virus in the absence of salts.

Virus	Original HA	Supernatant HA		Treated cells	
		First	After RDE	A.S.C.	Meconium agglutination
A. H.LEE dialysed + glucose	400	300	<1	<2	<10
B. H.LEE undialysed allantoic fluid	500	70	40	32	40,000

Meconium agglutination: Dilution to which a standard solution of semi-purified meconium inhibitor agglutinates cells treated as described in the text.

A.S.C. (agglutinin on stable cells, Anderson, 1947): Dilution of a 1 p.c. suspension of treated cells which when mixed with 1 p.c. normal fowl cells will give partial agglutination of the mixture.

Human cells were treated with the appropriate amount of periodate, spun down and then washed with 5 p.c. glucose solution (with 0·001 M phosphate). They were divided into two portions, one (A) being treated with dialysed H.LEE virus to which glucose had been added to 5 p.c., the other (B) with normal allantoic fluid H.LEE virus. No agglutination occurred in A, the usual gross agglutination in B. After centrifugation the supernatant fluids were titrated and the cells washed with glucose phosphate solution. They were then suspended in CA saline and treated for 30 minutes at 37° C. with 2,000 units of purified RDE. The supernatant fluids were titrated for haemagglutinin (HA) and the cells washed with citrate saline and made up to a 1 p.c.

suspension for the standard tests of PVR cells. The results are shown in Table 3.

The failure to adsorb H.LEE virus is clearly evident. Similarly prepared periodated cells in glucose were tested in serial NaCl dilutions in glucose, against cells treated in parallel with glucose alone, for agglutinability by active and indicator viruses MEL and LEE. The end-points were precisely the same for periodated and normal cells.

Periodate treatment does not therefore allow adsorption of virus in the absence of salts and the difference from normal cells must be in some secondary process.

Influence of ions on receptor destruction by virus.

In none of the experiments on adsorption and reliberation of virus was it possible to obtain complete absence of adsorption of virus in the presence of the lowest ion concentrations compatible with the presence of satisfactory cells. No sign of macroscopic agglutination was visible in these experiments: what adsorption occurred must therefore have been of insufficient intensity to allow "bridging" between cells. It has also been found that the phenomenon of irreversible union of indicator virus to fowl red cells can take place at 37° C. in very low salt concentrations (Burnet, 1952) without visible agglutination occurring. These findings made it desirable to study the influence of ions on the enzymic destruction of red cell receptors. It was found that using the standard dialysed viruses MEL and LEE, fowl cells initially agglutinated (i.e. in the presence of 0·01 M or higher concentrations of salt) became stable by spontaneous elution of virus in 2–2½ hours at 37° C. With lower concentrations of salt no agglutination occurred but destruction of receptors was evident in all including the preparations containing only the amount of ions unavoidably present in the reagents. The following experiment may be cited to establish the presence of receptor destruction without addition of salts.

Fowl cells were prepared in 5 p.c. glucose solution to which sodium phosphate (pH 8) had been added to a concentration of 0·002 M in terms of Na^+, a 20 p.c. suspension of cells being used as stock. Glucose in the form of a 50 p.c. solution was added to virus dialysed for two days in the cold against 0·001 M borate, to bring the concentration of glucose to 5 p.c. 2·25 ml. of virus MEL (HA titre 640) or LEE (HA titre 500) was mixed with 0·25 ml. of 20 p.c. cells in glucose solution. The tubes were held in a waterbath at 37° C. for 2½ hours with occasional shaking. No agglutination occurred at any stage.

The cells were then spun down, the supernatant fluid retained for titration and the cells washed twice with glucose + 0·002 M phosphate solution, in order to ensure removal of all free virus before the cells were brought into contact with saline. A 1 p.c. suspension in saline was then prepared and tested for stability. LEE treated cells were stable but MEL gave cells giving slightly

over one plus agglutination. Portion of each set of cells was therefore treated with dilute (1:100) immune ferret serum of homologous type after which the cells were washed and made up to 1 p.c. in saline. The cells were now tested against 5 agglutinating doses of the viruses NDV, MEL and LEE to determine the degree of receptor destruction.

TABLE 4.

Destruction of receptors in the absence of added salts.

Treatment of cells	NDV	MEL	LEE	Control
MEL	+	+	++	+
MEL + serum	—	—	++	—
LEE	—	—	—	—
LEE + serum	—	—	—	—
Normal cells	++	++	++	—

Table 4 shows that NDV and MEL are no longer capable of agglutinating cells treated with MEL and that LEE treated cells are inagglutinable by all three viruses. With both viruses there was a significant loss of haemagglutinin titre in the tubes containing red cells as against a control of virus plus glucose held at the same temperature, MEL 250 against 500 in the control and LEE 320 as against 450.

The action of the viruses does not proceed quite so far nor as rapidly as when a normal quantity of ions (0·10 M NaCl) is present in the preparation but the point to be stressed is that a normal degree of receptor destruction is possible under conditions where no agglutination takes place at any temperature and where absorption in the cold is minimal.

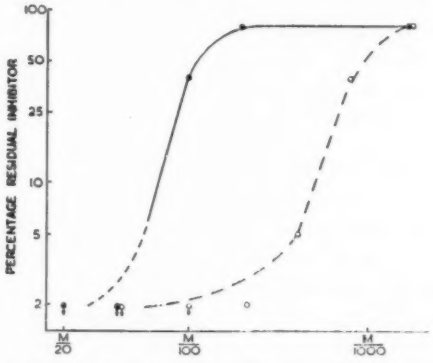

Fig. 4. To show the influence of salts on the union of indicator virus with meconium inhibitor.

The graphs show the percentage of haemagglutinin free after contact with inhibitor at various salt concentrations.

●——● NaCl ○– – –○ $CaCl_2$

Influence of salt concentration on union of indicator virus and inhibitor.

These experiments were based on the fact that contact between inhibitor and indicator virus results in a fairly slow progressive increase in the amount of virus inhibited. If a suitable mixture is chosen which after the standard 30 minutes' contact at room temperature in the presence of the usual amounts of salts is just

completely inhibited, then by making the same mixture in varying salt concentrations and then titrating immediately evidence of any slowing of the rate of union with lower salt concentrations should be obtained.

In the experiment whose results are depicted in Fig. 4 meconium inhibitor was diluted 1:20 in appropriate salt solutions. An equal volume was added to dialysed virus H.LEE previously diluted with an equal quantity of distilled water. After standing 30 minutes at room temperature (22° C.) the mixtures were serially diluted in tubes containing 0·25 ml. of 0·5 p.c. fowl cells in saline. The dilutions to which haemagglutination extended are plotted as ordinates in the Figure. It will be seen that very little inhibition at all is shown in the absence of added salt and that, as usual, Ca^{++} is more effective than Na^{+} in allowing union of inhibitor with indicator virus.

Results with T.MEL were essentially the same as those obtained with H.LEE.

Enzymic action of influenza virus in relation to ions.

It was shown some years ago that Ca^{++} was necessary for the action of RDE either on red cell receptors or soluble mucoids (Stone, 1947; Burnet, 1948) and had a significant effect in increasing the enzymic activity of influenza viruses on soluble mucoids. This past evidence of the importance of ions in these reactions was in part the stimulus to the more detailed work done in the present investigation.

The results obtained indicate that no action whatever takes place in the complete absence of ions, that Ca^{++} is much more effective than Na^{+}, and that in high ionic concentrations the reaction is again inhibited and may show important qualitative changes.

TABLE 5.

Comparative inhibitor titrations by PVR cells and standard method.

Inhibitor	Titre with PVR cells				Standard titration inhibitor	
	MEL	T. MEL	LEE	H. LEE	T. MEL	H. LEE
Meconium	400	1,000,000	<100	640,000	12,000	5,000
Ovomucin	<100	60,000	<100	36,000	10,000	5,000

In view of the use that has been made of PVR cells carrying indicator viruses in this investigation it is probably desirable to put on record the behaviour of such cells with the two inhibitors commonly used in this laboratory, viz. ovomucin (Gottschalk and Lind, 1949) and meconium (French, Curtain and Pye, 1952). Titrations of standard stock solutions were made with PVR cells carrying respectively MEL, T.MEL, LEE and H.LEE viruses. Table 5 shows

that the titre indicated by PVR cells is about 100 times higher than that obtained in standard inhibitor titrations with meconium, the discrepancy being not so great but still large with the ovomucin.

The results of experiments on the influence of ions on the enzymic action of MEL and LEE viruses may best be indicated by a series of representative graphs in which percentage of residual inhibitor is plotted as ordinate against salt concentration. In all experiments a preparation of semipurified meconium was used as inhibitor, the technique being as described under methods.

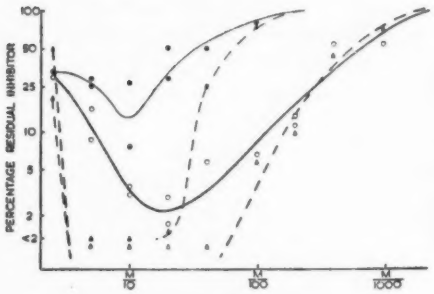

Fig. 5.

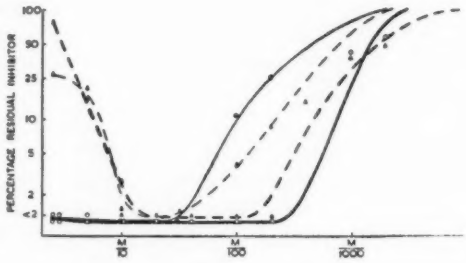

Fig. 6.

Fig. 5. Enzymic action of MEL on meconium inhibitor at varying NaCl and $CaCl_2$ concentrations.
The graphs show the percentage of residual inhibitor as titrated by

● H. LEE, after action in NaCl
○ H.LEE, after action in $CaCl_2$
▲ T. MEL, after action in NaCl
△ T. MEL, after action in $CaCl_2$

Fig. 6. Enzymic action of LEE on meconium inhibitor. Symbols as in Fig. 5.

Figs. 5 and 6 show respectively the action of dialysed viruses MEL and LEE, inhibitor being titrated only by the standard method against the indicators T.MEL and H.LEE.

MEL action shows the typically greater action on T.MEL inhibitor than on H.LEE inhibitor. Action on the heterologous receptor is considerably greater in Ca^{++} than in Na^{+} and the optimal Ca^{++} concentration is 0·05 M as against 0·10 M in Na^{+}.

LEE is a more active virus enzymically and destroys both inhibitors over the active range of salt concentrations. With higher concentrations of either Na^{+} or Ca^{++} we see the phenomenon of reversal of the inhibitor gradient, i.e. the capacity to destroy T.MEL inhibitor is lost although H.LEE inhibitor disappears.

In the lower salt concentrations titrations of residual inhibitor by agglutination of PVR cells run closely parallel to those carried out by the standard

method. In higher salt concentrations, however, there are significant dissociations which are exemplified in Figs. 7 and 8, showing details of MEL and LEE action in high concentrations of $CaCl_2$.

It will be seen that the diminution of enzymic action by high salt concentration, which is similar to that already noted by Gottschalk and Lind (1949) with ovomucin, is shown more markedly by tests with the heterologous indicator virus and by PVR cell tests in comparison with direct inhibition of haemagglutination tests. The most illuminating discrepancy is in regard to the action of LEE where meconium treated in M/2·5 $CaCl_2$ shows almost full power to agglutinate PVR-H.LEE cells but has no inhibitory effect against haemagglutination by H.LEE. It is posible that a small inhibitory titre is masked by Ca^{++} agglutination of the red cells but this effect is slight when normal saline is present and cannot account for more than a fraction of the discrepancy.

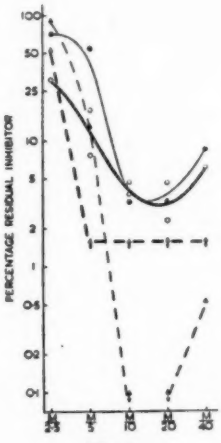

Fig. 7.

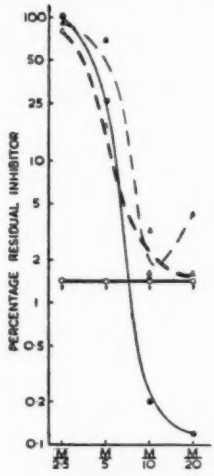

Fig. 8.

Fig. 7. Detail of enzymic action of MEL on meconium inhibitor at high concentrations of $CaCl_2$.

○——○ Standard inhibitor titration with H.LEE.
△——△ Standard inhibitor titration with T.MEL.
●——● PVR cell agglutinin titre with H.LEE.
▲——▲ PVR cell agglutinatinin titre with T.MEL.

Fig. 8. Detail of enzymic action of LEE on meconium inhibitor in high concentrations of $CaCl_2$. Symbols as in Fig. 7.

It was shown by Edney (1949) that "calgon" (sodium hexametaphosphate) had a considerable but far from complete inhibitory effect on the enzymic action of LEE on an inhibitor cyst-mucoid, very similar to meconium inhibitor. The experiments were done with 0·017 M calgon. This finding has been confirmed in the present work. Calgon was used at 0·0067 M, equivalent to about 0·04 Na^+ which with LEE allows maximal action on meconium inhibitor when in the form of NaCl. The results obtained under the same conditions as in the other experiments are shown in Table 6.

It may be noted that 0·0067 M calgon in glucose solution allows full adsorption of virus in the cold and that haemagglutination tests carried out with ·033 M calgon in distilled water as sole diluent give normal titres. Similarly PVR-H.LEE cells are agglutinated by meconium to slightly higher titre

(2,000,000) in that diluent than in saline while with PVR-MEL cells the result was identical (200) in both diluents. These findings may be of importance in indicating that the ionic conditions for adsorptive reactions differ somewhat from those optimal for enzymic action.

TABLE 6.

Action of LEE virus as enzyme on meconium inhibitor in presence of "Calgon."

Treatment in presence of	Residual inhibitor against H.LEE	T.MEL
$CaCl_2$ M/20	<2	<2
Calgon M/150	25	80
Calgon M/150 + NaCl M/10	<2	18
Aq. D	30	120
No virus	30	160

DISCUSSION.

These results confirm and extend the findings of Lowell and Buckingham, and Davenport and Horsfall for red cells suspended in glucose and of Flick. Sandford and Mudd (1949) for cells rendered insusceptible to haemolysis by treatment with 50 p.c. formalin. All groups found that adsorption of virus and agglutination did not occur in the absence of ions.

Earlier work from this laboratory (Stone, 1947; Burnet and Stone, 1947; Burnet, 1948; Edney, 1949) was much concerned with the influence of Ca^{++} and Ca^{++} deionizing agents on the enzymic activity of the influenza viruses and RDE. The experiments were, however, almost all carried out in the presence of NaCl and other salts present in undialysed virus fluids.

The primary stimulus to undertake a new study of this field came from the paper by Puck, Garen and Cline (1951) on the influence of ions on the attachment of bacterial viruses to their host cells. They found for the phage T1 that a concentration of Ca^{++} or $Mg^{++}M/10^{3.2}$ was optimal, with NaCl $M/10^2$. With high Ca^{++} concentrations the attachment was inhibited. By treating cells to which virus had been attached in the cold with distilled water most of the virus was removed so that the early stages at least of the reaction are reversible. The authors refer to evidence not yet available to us that this is rapidly followed by irreversible union which has a temperature dependence curve resembling that of an enzyme controlled reaction. The general resemblance to the influenza virus-red cell system is obvious and it is tempting to apply some of their theoretical ideas to the present problem.

Puck and his collaborators consider that cations play a major part in primary union of virus to cell "receptor." In their view union consists for the first step at least of an electrostatic attachment between points of opposite

polarity distributed in complementary fashion over the reacting surfaces. The rôle of inorganic ions is to be attached to specific sites on the two bodies (and presumably on the actual complimentary molecular groupings concerned) thereby furnishing the charge distribution necessary for virus cell attachment.

The findings with influenza viruses seem to indicate broadly that cations play a significant part in all reactions but that the primary adsorptive reactions are more strikingly influenced than the enzymic ones. Nothing that has emerged during the present study is incompatible with the general conclusion presented in two recent reviews (Burnet, 1951 a and b) that all the reactions centre round the interaction of enzyme groupings E on the virus surface and substrate groupings S which are distributed over macromolecules of mucoprotein character either on the cell surface or in solution as mucoid inhibitors. The indicator state was regarded as due essentially to a change of E to E^1 in which its specific adsorptive pattern is retained but the power to activate and disrupt the substrate is lost. Similarly by limited periodate action S can be converted to S^1 which is still susceptible to RDE action but not to the enzymic action of the isodynamic enzymes present on influenza viruses.

Without attempting to interpret all the detail that has been presented in this paper we can follow Puck in assuming that cations are specifically adsorbed to E or E^1 and serve as the primary means of bringing E-S apposition. It is only when several such unions are involved between virus particle and the red cell surface that the characteristic phenomena of absorption and haemagglutination can be observed; primary union does not in itself give rise either to enzymic action or to the type of firm union with indicator virus described in the accompanying paper. Primary union can be regarded simply as a preliminary apposition of E to S which makes eventual correct mutual orientation possible. When this occurs E can make specific activating union with S and disrupt it while E^1 can form the equivalent *stable* enzyme^1 substrate compound with either S or S^1. The influence of calgon on virus enzymic action suggests rather strongly that virus even after dialysis carries Ca^{++} firmly integrated into E but there is no conclusive evidence that Ca^{++} cannot be replaced by Na^+.

SUMMARY.

In the absence of ions none of the adsorptive or enzymic interactions of influenza viruses with mucoid inhibitors or cell receptors occur.

Visible agglutination is seen down to a dilution around 0·01 M for monovalent cations and active viruses. Indicator viruses react to a lower concentration of salts.

All the evidence points to the cations being the important factor in the phenomena; sodium and potassium are approximately equivalent, calcium is the most potent, especially in allowing enzymic action. The only anionic effect

clearly demonstrable is an inhibitory effect of sodium hexametaphosphate which is probably an anti-calcium action.

Adsorption of virus by red cells in the cold is most effective at relatively high salt concentrations but there is no sharp threshold and some adsorption takes place at the lowest practicable salt concentrations.

Destruction of red cell receptors by virus takes place at 37° C. at salt concentrations much below those required to produce visible agglutination.

Union of indicator virus and mucoid inhibitor at room temperature requires the presence of salts, Ca^{++} being more effective than Na^{+} in approximately the same ratio as is observed for enzymic action.

No enzymic action of virus on a typical mucoid inhibitor occurs in the absence of salts. Ca^{++} allows action at a level approximately 1/10 of that required with Na^{+}.

At high salt concentrations there is an inhibitory effect on enzymic action which may also be associated with a reversal of the inhibitor gradient.

The use of PVR cells for direct titration of inhibitors is a valuable and highly sensitive technique. Under certain conditions involving the presence of high salt concentrations titres may be widely different from those given by the standard inhibitor titration.

Periodate treated red cells in the stage of "modification" have the same salt requirements for agglutination as normal cells and are equally susceptible to the action of RDE.

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IRREVERSIBLE BINDING OF INFLUENZA VIRUS TO THE RED CELL SURFACE

by F. M. BURNET[1]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

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In previous work from this laboratory it has been shown that mumps (Burnet, 1946) and Newcastle disease viruses (Burnet and Anderson, 1946) can be irreversibly attached to red cells. This was established by showing that the cells after treatment with the virus at 37° C. were agglutinable by specific anti-viral immune sera and retained this agglutinability after treatment with receptor destroying enzyme (RDE) prepared from cholera vibrio cultures. Other properties of NDV treated cells, notably their ability to agglutinate normal fowl cells, were described by Anderson (1947).

A somewhat similar type of virus cell union was evident in Fazekas de St.Groth's (1949) experiments with periodate treated cells. Erythrocytes treated with an appropriate "modifying" amount of periodate were successfully exposed to virus and RDE. These "PVR" cells produced stable suspensions and the presence of virus firmly held on the surface could be demonstrated in several ways. PVR cells agglutinate normal cells, they are specifically agglutinated by immune sera against the virus used and when an inhibitor virus is used they are agglutinated by mucoid inhibitor solutions to high titre.

It has not previously been reported that an essentially similar type of union can be observed when indicator virus prepared from standard influenza strains is allowed to act on fowl red cells at 37° C. As the observation may have considerable bearing on the interpretation of the interaction between virus and cell receptor a brief account of the experiments and their implications is given in this paper.

The work arose as a result of experiments reported elsewhere (Burnet and Edney, 1952) on the influence of ions on virus cell union. When cells and virus are mixed in isotonic glucose solution with only minimal amounts of salt present no union occurs. It seemed possible, therefore, that adsorbed indicator virus (not spontaneously elutable) might be removed by replacing the normal saline suspending medium by glucose solution. It was found in fact that no release of virus occurred. To complete the experiment the cells were next transferred to a suitable saline medium containing calcium and acted on by RDE.

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Only about one-third of the expected amount of virus was liberated so experiments were made to see whether virus not removable by RDE could be demonstrated on the cells. The results closely resembled those obtained with PVR cells and further experiments were undertaken.

Materials and Methods.

The standard viruses MEL A and LEE B only were used. Dialysed allantoic fluid viruses were prepared as described in the accompanying paper (Burnet and Edney, 1952). Indicator virus was produced by simple heating of LEE to 56° C. for 30 minutes, MEL being converted by the addition of 0·0002 M calgon and adjustment of the reaction if necessary to pH 8·0 before heating. Ferret immune sera against MEL and LEE were diluted 1: 10 in saline containing 0·1 p.c. $CaCl_2$ and buffered with acetate at pH 6·2 (CA saline) and treated with 200 units of purified RDE for 2 hours at 37° C. to remove non-specific mucoid inhibitors. Meconium inhibitor solution was that used in the experiments reported in the accompanying paper. Details of procedure are more conveniently included in the main text.

EXPERIMENTAL.

The characteristics of red cells to which indicator virus is irreversibly adsorbed.

The reaction of cells to which influenza virus LEE in the indicator phase is irreversibly adsorbed can be exemplified by the following experiment. Allantoic fluid virus was dialysed for 48 hours against a large volume of 0·001 M borate solution in the cold room. It then had a haemagglutinin titre of 1,000. Portion of the dialysed fluid was heated for 30 minutes at 56° C., to convert the virus into the indicator phase. The haemagglutinin titre was now 600 and the virus had no enzymic action on dilute meconium inhibitor.

To 5 ml. of heated dialysed virus 0·75 ml. of 1·0 M NaCl solution and then 0·125 ml. of packed fowl erythrocytes were added, mixed well and placed in a waterbath at 37° C. for an hour. Rapid gross agglutination occurred and the tube was shaken occasionally during the course of incubation. After one hour the cells were spun down and the supernatant fluid replaced by 5 ml. of CA saline pH 6·2. 0·25 ml. of a solution of purified RDE, titre 8,000, was added and the cells exposed to its action at 37° C. for 1 hour with occasional shaking. Progressive stabilization of the cells could be seen. The mixture was centrifuged, the supernatant fluid discarded and the cells washed once with 5 ml. of 2 p.c. sodium citrate saline. Finally, they were made up to a 1 p.c. suspension in saline to which approximately 0·1 p.c. of sodium citrate was added.

In the ASC test (agglutinin on stable cells—Anderson, 1947) twofold dilutions of the 1 p.c. cells in saline were prepared and an equal 0·25 ml. volume of normal 1 p.c. fowl cells added. On settling the cells showed agglutination with a partial "one-plus" endpoint when the 1 p.c. treated cells had been diluted 1: 24 before the second volume of normal cells was added. Both

the treated and the normal cells were stable separately. Slight traces of granularity round the central button of deposited cells were usually visible with treated cells but if the degree did not exceed our conventional ± level the cells were regarded as stable.

As shown by Fazekas de St.Groth (1949) PVR cells coated with H.LEE virus are agglutinated by appropriate indicator mucoids and a similar test was applied to our treated cells. Tenfold dilutions of a semipurified and dialysed preparation of meconium inhibitor were prepared in saline and to 0·25 ml. volumes an equal volume of 1 p.c. treated cells was added. Finely granular complete agglutination was produced to a 10^{-5} dilution of the stock (±0·4 p.c.) solution with partial agglutination at 10^{-6}.

Treated cells were also agglutinated by animal sera to very high titres but when immune ferret sera were diluted in CA saline and treated with RDE, specific agglutination to a low (150) titre was given by LEE antiserum and not by MEL antiserum.

In summary the treated cells behave as if they had firmly attached to their surface virus particles which are still capable of union with intact receptors on other cells, inhibitor mucoids and specific antibody. These virus particles cannot be removed by RDE immune serum or inhibitor mucoid. Subsequent experiments were directed toward establishing the conditions under which the phenomenon occurred.

If the same procedures are gone through with unheated LEE virus either in the form of original allantoic fluid or after dialysis there is no evidence of more than minimal amounts of virus being attached. However, one never gets completely negative ASC tests, probably indicating that a proportion, perhaps 5–10 p.c. of cells, have one or more firmly attached virus particles.

Time and temperature requirements for irreversible union.

In the experiments to be reported 1 ml. of virus (to which 0·15 M NaCl had been added if dialysed material was used) was mixed with 0·25 ml. of a 20 p.c. suspension of fowl erythrocytes. After exposure to the required temperature for the appropriate period the tubes were centrifuged and the supernatant fluid replaced by 1 ml. of CA saline and 0·1 ml. of purified RDE added. After 1 hour at 37° C. the cells were spun down, washed with 2 ml. of 2 p.c. citrate saline and made up to 1 p.c. suspensions with normal saline. The cell suspensions were now tested (1) for stability in an equal volume of normal saline; (2) for capacity to agglutinate normal fowl cells (ASC titration); (3) for agglutinability by meconium inhibitor solution and (4) for agglutinability by RDE-treated immune sera. The results shown in Table 1 indicate that the development of firm union with fowl cells proceeds much more rapidly at higher temperatures and requires about 30 minutes' contact at 37° C.

to reach completion. It is evident also from the Table that with inadequately treated cells the higher concentrations of meconium inhibitor have very little agglutinating effect.

TABLE 1.

Time and temperature effects with H. LEE virus.

Time	Temperature (°C.)	ASC	Meconium agglutination					Stability	Agglutination with anti-LEE serum
			10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
60′	0°	5	—	—	—	—	—	—	—
5′	21°	5	—	—	—	±	—	—	—
15′	21°	7	±	+	++	++	±	—	—
30′	21°	10	+	+	++	++	±	—	—
5′	37°	7	±	+	++	++	±	—	—
15′	37°	12	+	++	++	++	+	—	+
30′	37°	20	++	++	++	++	+	—	++
60′	37°	20	++	++	++	++	+	—	++

Essentially similar findings were obtained with indicator MEL virus prepared by heating in the presence of 0·0002 M calgon but with this strain union was established within 15 minutes at 37° C.

Relation of capacity for irreversible union with the indicator state.

Dialysed virus LEE was heated for 30 minutes over the range of temperatures from 46° to 64° C. and the heated virus tested for its capacity to give irreversibly coated cells and for its enzymic action on meconium inhibitor. Fowl cells were treated according to the technique already described and tested for stability, ASC, agglutinability by meconium inhibitor and by RDE-treated immune serum. The enzymic action was tested as follows: mixtures were prepared containing in a final volume of 1·25 ml., 0·25 ml. of heated virus, 0·25 ml. of a 1 : 100 dilution of stock meconium inhibitor, and 0·75 ml. of CA saline. The tubes were held at 37° C. in a waterbath for one hour and the haemagglutinin then destroyed by heating in a boiling water bath for two minutes. Residual inhibitor was titrated by its capacity to agglutinate H.LEE cells. Table 2 shows that the capacity to produce firm union is associated with absence of enzymic action on the inhibitor mucoid in parallel with the earlier demonstration (Burnet, 1948) of the similar relationship between indicator activity and loss of enzymic action.

The results of a similar experiment with MEL are shown in Table 3.

It will be seen that the dialysed virus used in this experiment was not converted into indicator by simple heating at 48°–56° C. and showed only minimal capacity for irreversible union. On the other hand heating in the presence of calgon showed partial conversion to indicator at 40° C.

TABLE 2.

Effect of heat on virus LEE in inducing capacity for irreversible union in relation to loss of enzymic activity.

Temperature (°C.)	Time	Haemagglutinin titre	Character of treated F cells			Enzymic activity
			Stability	ASC	Meconium agglutination	
46°	30′	700	—	3	00000	>3
48°	30′	700	—	8	10000	>3
50°	30′	600	—	24	22210	3
52°	30′	600	—	20	22220	0·5
54°	30′	600	—	20	22220	0·5
56°	30′	600	—	20	22220	0
58°	30′	600	—	20	22220	0
60°	30′	600	—	20	22220	0
62°	30′	600	—	20	22220	0
Unheated		800	—	<2	00000	>3

Meconium agglutination. Agglutination of cells by meconium dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 2 = complete, 1 = partial, 0 = trace or no agglutination.

Enzymic activity = $\log_{10}$ diminution in agglutinin titre of a 1 : 500 meconium inhibitor solution after one hour's action of virus at 37° C.

TABLE 3.

Effect of heat on virus MEL in inducing capacity for irreversible union.

Heated for 30′ at (°C.)	Haemagglutinin titre	Enzymic activity	Indicator	Character of treated F cells		
				Stability	ASC	Meconium agglutination
56°	320	1·7	<2	—	8	10000
50°	400	1·7	<2	—	8	10000
48°	700	1·7	<2	—	4	00000
56° + Calgon	180	0	3·7	—	14	22220
50° + Calgon	250	0	3·7	—	14	22220
48° + Calgon	250	0	3·7	—	20	22220
46° + Calgon	400	0·4	3·7	—	20	22220
44° + Calgon	400	0·2	3·7	—	20	22220
40° + Calgon	400	0·4	2·3	—	8	22100
37° + Calgon	400		<2	—	10	12200
Unheated	800	2·7	<2	—	<2	00000

Indicator. $\log_{10}$ dilution of meconium solution showing endpoint agglutination. <2 indicates virus is not in indicator phase.

In both experiments it is noteworthy that the capacity for irreversible union develops slightly before the full development of the indicator state.

Ionic requirements for production of irreversible union.

It is of considerable interest that the ionic requirements for the type of union we are considering seem to be far lower than for the various adsorptive reactions discussed in the accompanying paper. Using reagents rendered iso-

tonic with glucose with minimal amounts of sodium phosphate and borate but with no added salt, cells showing typical reactions could be obtained with both H.LEE and T.MEL.

Dialysed virus LEE and MEL were converted into indicator by heating at 50° C. for 30 minutes, MEL after the addition of 0·0003 M calgon. Glucose was added to bring the concentration to 5 p.c. Fowl cells were prepared in glucose containing 0·002 M phosphate. To 1 ml. of each virus 0·25 ml. of 20 p.c. glucose cells was added; in one set of tubes 0·05 ml. 1·0 M NaCl was added, the other contained no added salt.

The mixtures together with control glucose-virus without cells, were held at 37° C. for an hour. Gross agglutination occurred in the tubes containing NaCl, none in the others. The cells were spun down and the supernatant fluids removed for titration. The deposited cells were resuspended in glucose phosphate solution and were held at room temperature for 10 minutes to establish that no agglutination of the NaCl cells occurred. The tubes were then respun and the washed cells treated in the usual fashion with RDE. Table 4 shows that despite the absence of agglutination there was a disappearance of the greater part of the haemagglutinin from the supernatant fluids of the mixtures without added salt. After treatment with RDE the cells were slightly unstable but gave perfectly adequate evidence that the virus had been firmly adsorbed.

TABLE 4.

Production of irreversible union with very low salt concentration.

Virus	Added salt	Supernatant haemagglutinin titre	Treated cells		
			Stability	ASC	Meconium agglutination
H.LEE	NaCl 0·04 M	4	±	16	22221
H.LEE	0	60	+	10	22211
Control		480			
T.MEL	NaCl 0·04 M	<2	+	16	22221
T.MEL	0	12	±	14	22221
Control		480			

Virus concentration, pH and other factors.

The experiments so far reported have all involved the use of about 0·05 ml. of packed cells with undiluted virus having a haemagglutinin titre after dialysis and conversion to indicator in the range 300–600. Tests to see what extent dilution is permissible if typically changed cells are to be obtained are illustrated in Table 5. In this experiment a typical dialysed and heated LEE fluid was diluted serially in CA saline and 1 ml. of each dilution used to treat 0·05 ml. of packed fowl cells.

The effect of heated dialysed LEE virus on fowl cells is not detectably

influenced by pH within the range 6·0–8·0. Systems adjusted to pH 6·0, 7·0 and 8·0 were prepared by the addition of 0·02 M sodium phosphate buffer. The cells obtained gave identical reactions.

TABLE 5.

Influence of virus concentration on cell treatment with H.LEE virus.

Virus dilution	Haemagglutinin Titre	Character of treated cells	
		ASC	Meconium agglutination
Undiluted	500	16	22221
1 : 3·2	160	7	22221
1 : 10	50	3	00100
1 : 32	16	<2	00000
1 : 100	5	<2	00000

Under the standard conditions used for fowl cells, human red cells are incapable of binding irreversibly either LEE or MEL indicator viruses. Cells treated with virus giving typical reactions with fowl cells showed low ASC titres, 3 with indicator MEL, 5 with indicator LEE and no trace of agglutination by meconium inhibitor.

Necessity for intact receptors to allow irreversible union.

By analogy with other virus cell reactions, pretreatment of the red cells with RDE should prevent irreversible union of the present type. That this is the case can be shown by the following experiment in which 3 identical mixtures of virus, cells and RDE were used, differing only in the order in which the reagents were allowed to interact.

Each tube finally contained 1 ml. dialysed H.LEE virus to which 0·15 ml. 1·0 M NaCl had been added (V), 0·05 ml. packed fowl cells (C), 0·1 ml. purified RDE (titre 8,000), and 0·5 ml. CA saline (CAS). The sequence of additions was:

(1) C, CAS, RDE–60′ at 37° C.–V–60′ at 37° C.
(2) C, CAS, V–2′ at 21° C.–RDE–118′ at 37° C.
(3) C, CAS, V–60′ at 37° C.–RDE–60′ at 37° C.

After the full two hours had been completed at 37° C. the tubes were centrifuged, the cells washed once with citrate saline and tested as usual. The results are shown in Table 6.

TABLE 6.

Failure of reaction with cells pretreated with RDE.

Sequence of treatment	Reactions of treated cells		
	Stability	ASC	Meconium agglutination
1. C, RDE — V	—	<2	00000
2. C, V, RDE	—	10	22220
3. C, V — RDE	—	32	22221

It is of interest that only a short exposure to virus before the addition of RDE is needed to allow the cells to take up sufficient virus to give typical reactions.

DISCUSSION.

These experiments may be summarized as demonstrating a previously undescribed type of union of influenza virus to the fowl erythrocyte surface, a union which is not reversible by the action of RDE, immune serum or mucoid inhibitor. For such irreversible union to occur the necessary conditions are:

(1) That the virus is in the indicator state, i.e. still capable of agglutinating red cells but lacking enzymic activity and inhibited by mucoid inhibitors.

(2) That the virus and cells react at 37° C. for a time of the order of 15–30 minutes; at lower temperatures longer times are needed.

(3) That the cells have not previously lost receptors by RDE action.

(4) That an adequate amount of virus is available for the number of cells used.

From the point of view of theoretical interpretation, the points to be stressed are first, that RDE acting before addition of the virus prevents union but is inert *after* virus action; second, that the primary union of the virus to the cell causing rapid agglutination at any temperature between 0° and 37° C. does not in itself constitute irreversible union; a relatively slow secondary process is necessary. Two negative findings are also important, viz. that the phenomenon is not observable with human cells nor with enzymically active virus.

It must now be accepted that the virus-mucoprotein reactions, with which we have been concerned in this laboratory since 1945, centre around enzyme-substrate interaction, the enzyme (E) being in the form of active groupings distributed over the virus surface and the substrate (S) representing groups attached to mucoprotein or mucopolysaccharide macromolecules but not forming part of the essential architecture of the macromolecule nor concerned with its antigenic specificity. It is equally evident that the conditions of E–S interaction are greatly influenced (a) by the ionic environment and (b) by the atomic and electronic configurations adjacent to E and S groupings respectively.

Without attempting to traverse again all the evidence which has been discussed in two recent review articles (Burnet, 1951 a and b) we can see no escape from the conclusion that although indicator virus is lacking in any demonstrable enzymic activity, yet the E groups retain their essential adsorption relation to S although they lack the capacity to produce enzymic destruction of S. We can denote them as E^1.

The chief implication of the work described in this paper is that it points to the existence of two well defined stages in the union of E to S or E^1 to S.

There are hints of more than two stages but for simplicity only the two main ones will be discussed. The first is common to both active and indicator virus, it takes place rapidly in the cold or at any temperature up to 40° C. and it demands a considerable ion concentration in the medium. It is essentially reversible and almost certainly represents a dynamic equilibrium which only develops binding power sufficient to give demonstrable red cell agglutination when virus and cell (or inhibitor macromolecule) are united by two or more such unions. Virus in the first stage of union can be liberated from red cells by RDE by immune serum and, with some indicator viruses at least, by a soluble inhibitor.

The first stage is regarded as an unorientated attraction between E and S mediated in accordance with Puck's (1951) hypothesis by cations specifically adsorbed in defined sites on one or other reagent, most probably on the enzyme.

The second stage or more correctly the final stage is one proceeding relatively slowly at 37° C. and much more slowly at lower temperatures, and with lower ionic requirements. With active virus it takes the form of receptor or substrate destruction—except in the case of NDV and mumps where a proportion of the second stage unions give rise to irreversible union and haemolysis. With indicator virus irreversible union of the type described in this paper is produced.

The final stage is regarded as the establishment of a correctly orientated apposition of E to S resulting typically in enzyme-substrate union with activation and destruction of S with reliberation of E. Where E^1 and S are concerned the completion of chemical union gives an irreversible union with an attachment of virus to the substrate carrier that can only be broken by gross physical forces.

It seems highly probable that an intermediate stage of relatively firm orientated adsorption exists in which for one reason or other the final chemical union is delayed. This may be specially characteristic of some types of inhibitor-indicator virus union and is the most likely explanation of the failure of human cells to show the reaction.

SUMMARY.

When fowl erythrocytes are treated with indicator viruses H.LEE and T.MEL for some time at 37° C. a proportion of the virus becomes irreversibly attached to the cell surface. This can be demonstrated by three techniques: (a) the capacity of the cells to agglutinate normal cells; (b) agglutination of treated cells with soluble inhibitors, and (c) agglutination by immune serum previously treated with RDE. The phenomenon is not shown with active viruses nor with human red cells.

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INFLUENCE OF IONS ON THERMAL INACTIVATION AND MODIFICATION OF AN INFLUENZA VIRUS

by MARGARET EDNEY[1] AND F. M. BURNET[2]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

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Since the work of Francis in 1947, it has been known that heating the influenza virus B strain LEE at 56° C. for 30 minutes renders its haemagglutinin highly sensitive to the inhibitory action of a variety of mucoids. While the haemagglutinating activity of the unheated virus is not influenced by a stock solution of ovomucin (Gottschalk and Lind, 1949), that of heated virus is greatly diminished, five agglutinating doses being inactivated by dilutions up to 1 : 5,000 of the ovomucin. This change in character whereby the haemagglutinin becomes susceptible to inhibition by typical mucoids, is referred to as the development of the indicator state, and treated allantoic fluid with this character as indicator virus.

The influenza A strain MEL is not converted to the indicator state by simple heating, but Stone (1949) was able to transform the virus to a sensitive indicator by heating it in the presence of sodium citrate or calgon (sodium hexametaphosphate), both strong calcium-deionizing agents. This procedure is applicable to most influenza viruses. In the case of LEE, indicator virus always lacks demonstrable enzymic action on the mucoids that inhibit it. With influenza A strains the negative correlation between enzymic activity and effectiveness as an indicator is not as strict as with LEE and definite exceptions to the rule that an indicator has no enzymic action have been described by Smith and Westwood (1950) and Stone and Fazekas de St.Groth (in preparation).

In the course of general work on the influence of ions on influenza virus action (Burnet and Edney, 1952) the part played by ions in the transformation of MEL to the indicator state was studied. This paper reports experiments on the heating of MEL in various ionic media, and the changes brought about in the haemagglutinin titre, enzymic activity and indicator property of the virus by this treatment. Only one virus strain, MEL, was studied and although other influenza A strains may well behave similarly it should be stressed that the conclusions are applicable to this strain only. It should also be noted that less drastic conditions were required for conversion to indicator of dialysed virus than for crude allantoic fluid virus, i.e. lower temperature, or smaller concentrations of the appropriate ions.

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[1] Francis Haley Research Officer, Department of Experimental Medicine, University of Melbourne.

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Materials and Methods.

Virus strain.

The influenza A strain, MEL, was used in the form of infected allantoic fluid, harvested 2 days after inoculation of 11-day chick embryos. The fluids were dialysed for 48 hours through sacs made of "Visking" sausage casing against 100 volumes of 0·001 M borate buffer at 4° C. Exhaustive dialysis against distilled water resulted in great loss of haemagglutinin titre and the appearance of a precipitate; this was avoided by the use of the dilute borate buffer.

Borate buffer. pH 8·5 (Clark, 1920).

Phosphate buffers. Sodium phosphate buffers were prepared by mixing appropriate amounts of 0·10 M NaH_2PO_4 and 0·10 M NaOH to give a range of pH values. All buffers contained equivalent 0·10 M Na+ concentrations.

Calcium-acetate saline. Isotonic sodium acetate-acetic acid buffer adjusted to pH 6·2 and containing 0·1 p.c. calcium chloride.

Ovomucin. Ovomucin was prepared from egg-white by the method described by Gottschalk and Lind (1949).

Indicator virus T.MEL. T.MEL was prepared by a slight modification of the method of Stone (1949). MEL allantoic fluid virus was heated in the presence of 0·05 p.c. sodium citrate and 0·02 M borate buffer at 56° C. for 30 minutes. This is referred to as the standard treatment in citrate-borate.

Haemagglutinin (HA) titration. Serial two-fold dilutions of virus (0·25 ml.) were prepared in normal saline and an equal volume of 1 p.c. fowl red cells was added to each dilution. The cells were allowed to settle at room temperature and readings were made by observing the pattern of sedimented cells. The endpoint was taken as the amount of virus causing partial agglutination, this amount being one agglutinating dose (A.D.). The haemagglutinin (HA) titres are expressed as the reciprocal of the initial dilution of virus at the end-point.

Inhibitor titration (Burnet, 1948). Serial dilutions of inhibitor were prepared in normal saline. An equal volume of virus containing 5 A.D. was added and the mixtures kept for 30 minutes at room temperature. A third volume of fowl cells was added and these were allowed to settle at room temperature. Partial agglutination indicated the end-point.

Inhibitor-destroying titration (Stone, 1949.) Serial dilutions of virus were prepared in calcium-acetate saline and to each was added an equal volume of a standard dilution of ovomucin containing 5-10 inhibitory doses (each dose being capable of inhibiting agglutination by 5 A.D. of T. MEL). After incubation for 1 hour at 37° C. the mixtures were heated at 65° C. for 30 minutes to destroy the virus haemagglutinin, then cooled to room temperature. One drop of the indicator virus, T.MEL, containing 5 A.D. was added to each tube followed after 30 minutes at room temperature by one volume of 1 p.c. fowl red cells. Partial agglutination gave the end-point. The presence of agglutination indicates destruction of inhibitor and hence enzymic activity of the virus.

EXPERIMENTAL.

The general plan of these experiments was to heat the dialysed virus, diluted in various salts, at 56° C. for 30 minutes and test the changes brought about by this treatment in the properties of the virus. The haemagglutinin was first titrated against 1 p.c. fowl cells. The enzymic activity was measured by the *inhibitor-destroying titration* which gave a direct titre usually about half

the haemagglutinin titre for active dialysed MEL. The "indicator titre" was determined by measuring the inhibitory titre of ovomucin against 5 A.D. of the virus; it was thus possible to estimate indicator titre as long as the haemagglutinin titre was above 5. For the purposes of this paper the full indicator state was defined as that condition of the virus where the haemagglutinin is inhibited by a typical mucoid inhibitor (e.g. ovomucin) to a titre of the order shown by a typical indicator (e.g. T.MEL).

The presence of concentrations of salts in the range used in the experiments did not significantly affect these titrations. Virus mixed with salts, but not heated, gave the expected results; and virus dialysed again after heating gave similar titres before and after dialysis.

The results reported are representative of a number of experiments, but minor variations occurred between different batches of dialysed virus.

The effect of pH on thermal stability of dialysed MEL virus.

Preliminary experiments were done to determine the effect of pH on the thermal stability of virus in very low concentrations of ions. Dialysed MEL was diluted 1:5 in a range of phosphate and borate buffers (0·001 M with respect to Na^+) or, for the high pH range, in KOH (maximum concentration used 0·0013 M) and heated at 56° C. for 30 minutes. pH determinations were made by the aid of the glass electrode, corrected for alkaline error above pH 10 (Dole, 1941).

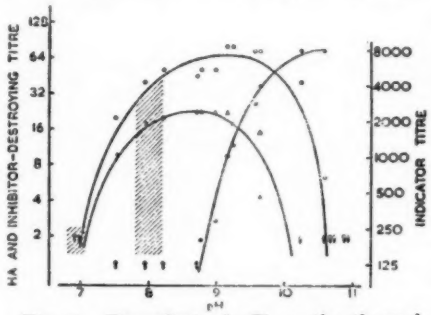

Fig. 1. The effect of pH on the thermal stability of dialysed MEL.

o Haemagglutinin (HA) titre.
△ Inhibitor-destroying titre-measuring enzymic action on ovomucin.
● Indicator titre, the inhibitory titre of ovomucin against 5 A.D. of the virus.

In order from left to right.

Titre of ovomucin with T.MEL as indicator = 8,000.
HA titre of virus before heating = 96.
Virus heated at 56° C. for 30 minutes.

Fig. 1 shows the results of haemagglutinin indicator and inhibitor-destroying titrations. Below pH 6·9 and above 10·6 the haemagglutinin and enzymic activity of the virus were destroyed. In the pH range 7–9 the inhibitor-destroying titre was proportional to the haemagglutinin titre, being about half. An unexpected result was the conversion of the virus to the indicator state with loss of enzymic activity when the pH was around 10.

Non-dialysed MEL virus was similarly converted to indicator, in a narrow pH range near the destruction point, by heating at 56° C. in 0·002 M KOH.

Two pH ranges (indicated in Fig. 1) were chosen for the study of the effect of addition of higher concentrations of ions to the diluting media.

1. About 6·9, where the haemagglutinin of dialysed virus was destroyed.
2. About 8, where approximately 50 p.c. of the haemagglutinin titre remained, the virus had normal enzymic activity and was not an indicator.

Experiments at pH 6·9.

At pH 6·9 it was found that addition of certain concentrations of salts to the virus protected the haemagglutinin from destruction by heating at 56° C.

Dilutions of the following Na salts were made in phosphate buffer (0·001 M): chloride, sulphate, citrate, hexametaphosphate. Dialysed MEL was diluted 1:5 in the salts and in the buffer alone, and the pH checked with universal indicator. In high concentrations of some salts, where the buffer was too weak to keep the pH constant, adjustments were made with small amounts of acid or alkali. After heating at 56° C. for 30 minutes, the haemagglutinin was titrated. The results are shown in Fig. 2 where percentage haemagglutinin titres are plotted against concentration of the salt expressed as $-\log_{10}$ of the molarity.

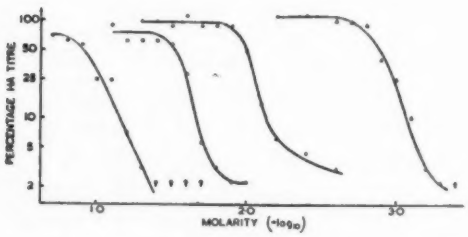

Fig. 2. Dialysed MEL heated at 56° C. for 30 minutes in sodium salts, at pH 6·9.

● Sodium chloride.
o Sodium sulphate.
▲ Sodium citrate.
△ Sodium hexametaphosphate (calgon).

In order from left to right.

The protective effect was evidently some function of the valency of the anion, increasing in the order: chloride, sulphate, citrate, hexametaphosphate. It was not linearly proportional to the valency at a given concentration, but in the case of NaCl and Na_2SO_4 it seemed to depend on the square of the valency, i.e. the ionic strength. The point where maximum protection ceased for NaCl was at a molarity of 0·10 (average of 3 experiments). For Na_2SO_4 the molarity was 0·03, normality 0·06 and ionic strength 0·10, very nearly the same as for NaCl.

Over the range of salt concentrations in which sufficient haemagglutinin remained to allow tests of its inhibition by ovomucin, it was found that the virus had been converted to indicator, although near the region of haemagglutinin destruction there was a variable retention of enzymic activity with incomplete conversion to the indicator state. Representative results for sodium chloride and calgon are given in Table 1.

TABLE 1.

The effect of heating virus at pH 6·9 in sodium chloride and calgon.

Salt	Molarity ($-\log_{10}$)	HA Titre	Inhibitor-destroying titre	Indicator titre
Sodium Chloride	·7	64	<2	8,000
	·9	53	<2	7,500
	1·1	24	2	4,300
	1·3	3	2	—
	1·5	<2	<2	—
Calgon	2·7	96	<2	8,000
	2·9	38	<2	7,000
	3·1	10	<2	4,000
	3·3	2	<2	—
	3·5	<2	<2	—

— Not tested because of low HA titre.
Titre of ovomucin with T. MEL as indicator = 8,000.
Buffer-phosphate pH 6·9.
Virus heated at 56° C. for 30 minutes.

Experiments at pH 7·8–8·2.

A more extensive series of experiments was undertaken in this range using borate (pH 8·2) or phosphate (pH 7·8) buffers. As has already been shown heating at 56° C. in dilute buffer (0·001 M) at this pH produced a moderate reduction in haemagglutinin titre without change to indicator. With increasing salt concentration there was first an increased destruction of haemagglutinin, the lowest value of haemagglutinin being reached for NaCl at about 0·025 M. With a further increase in salt concentration protection was almost complete (except in the case of $CaCl_2$ and $MgCl_2$), the zone of concentrations being approximately the same as that giving protection at pH 6·9. The highest salt concentrations used did not exceed 0·5 M so that a further zone of increased haemagglutinin destruction known to exist from limited experiments was not explored.

In Figs. 3a, 3b and 4 the percentage haemagglutinin titres after heating are plotted against the molarity of the salt used. The zones mentioned are clearly visible in all the curves with the exception of that for calgon.

Enzymic activity and indicator titre were measured as well as haemagglutinin titre in more detailed experiments on heating dialysed virus in dilutions of sodium chloride, sodium sulphate, sodium citrate and calgon (Figs. 5, 6, 7, and 8).

As before, in the "protected" zone, the viruses developed the typical indicator state with the disappearance of enzymic activity. In very dilute salts no such change occurred, the virus having the properties to be expected after being heated in the absence of added salts in this pH range. With citrate and calgon the changeover between these zones was well defined, occurring with the former

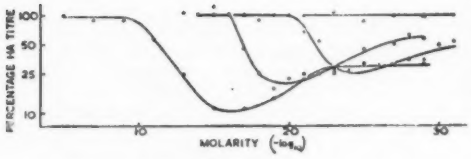

Fig. 3a.

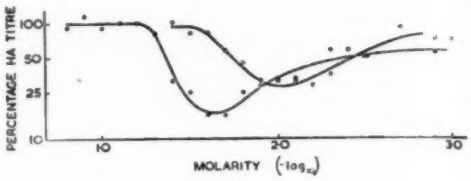

Fig. 3b.

Fig. 3a. Dialysed MEL heated at 56° C. for 30 minutes in sodium salts, at pH 8·2.

● Sodium chloride.
o Sodium sulphate.
▲ Sodium citrate.
△ Sodium hexametaphosphate (calgon).

Fig. 3b. Dialysed MEL heated at 56° C. for 30 minutes in sodium salts, at pH 8·2.

● Sodium nitrate.
o Sodium oxalate.

In order from left to right.

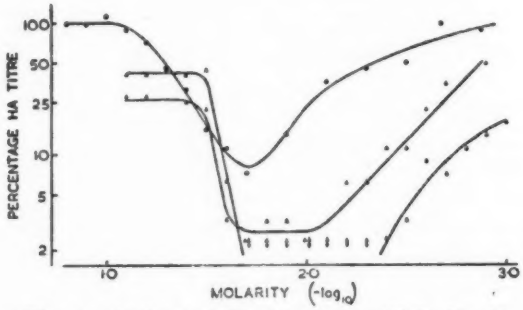

Fig. 4. Dialysed MEL heated at 56° C. for 30 minutes at pH 8·2 in

● Potassium chloride.
△ Calcium chloride.
▲ Magnesium chloride.

In order from left to right.

at a molarity of $10^{-3.0}$, and the latter $10^{-4.6}$. There were wider intermediate zones in the case of sodium chloride and sodium sulphate, where the viruses showed some enzymic activity and behaved as full or partial indicators.

Non-dialysed MEL virus, as was already well-known, became an indicator after heating in citrate and calgon, but in the highest concentrations of sodium sulphate and sodium chloride the change to the indicator state was only partial.

Heating in $CaCl_2$ at 52° C.

The importance of the removal of calcium ions in the production of indicator virus from strains refractory to simple heating has been stressed (Stone, 1949; Lanni, Lanni and Beard, 1950). The calcium-deionizing agents were the

most potent of the salts in converting MEL virus to indicator (Figs. 7 and 8) and in the case of PR8, Stone was able to bring about a partial reversal from the indicator to the non-indicator state by the addition of calcium ions.

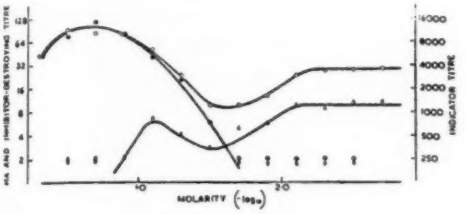

Fig. 5.

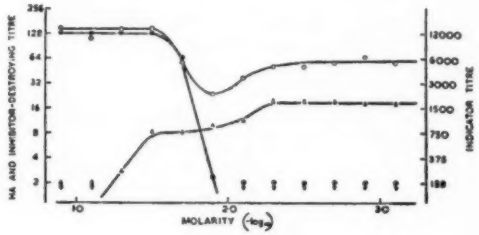

Fig. 6.

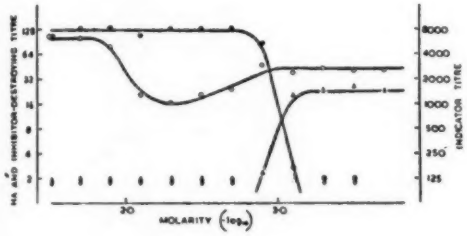

Fig. 7.

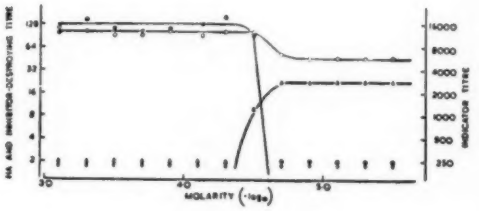

Fig. 8.

Fig. 5. Dialysed MEL heated at 56° C. for 30 minutes in sodium chloride.
● HA titre. o Inhibitor-destroying titre. △ Indicator titre.
Titre of ovomucin with T.MEL as indicator = 10,000. Buffer—borate pH 8·2.

Fig. 6. Dialysed MEL heated at 56° C. for 30 minutes in sodium sulphate.
● HA titre. o Inhibitor-destroying titre. △ Indicator titre.
Titre of ovomucin with T.MEL as indicator = 12,000. Buffer—phosphate pH 7·8.

Fig. 7. Dialysed MEL heated at 56° C. for 30 minutes in sodium citrate.
● HA titre. o Inhibitor-destroying titre. △ Indicator titre.
Titre of ovomucin with T.MEL as indicator = 8,000. Buffer—phosphate pH 7·8.

Fig. 8. Dialysed MEL heated at 56° C. for 30 minutes in calgon.
● HA titre. o Inhibitor titre. △ Indicator titre.
Titre of ovomucin with T.MEL as indicator = 12,000 Buffer—phosphate pH 7·8.
Virus heated at 56° C. for 30 minutes.
In order from left to right.

It was of interest to test the properties of MEL virus after heating in high concentrations of a calcium salt. Concentrations of $CaCl_2$ around 0·10 M (see Fig. 4) showed only partial protection of haemagglutinin after heating at 56° C. for 30 minutes and in higher concentrations the haemagglutinin was

destroyed. Hence dialysed MEL was heated at 52° C. for an hour, with dilutions of $CaCl_2$ made in distilled water, which seemed to be the most satisfactory diluent for preservation of haemagglutinin after heating in this salt. Controls were included of dialysed and non-dialysed MEL, treated in the standard way (citrate-borate) but at 52° C., and of T.MEL (heated at 56° C.). The results are shown in Table 2.

TABLE 2.

The effect of heating virus at 52° C. in calcium chloride.

Virus	Diluent	Temperature and time	HA titre	Inhibitor-destroying titre	Indicator titre
Dialysed MEL	$CaCl_2$ M/$10^{0.7}$	52° C. for 1 hour	53	<2	750
	M/$10^{0.9}$	52° C. for 1 hour	96	4	200
	M/$10^{1.1}$	52° C. for 1 hour	85	16	<100
	M/$10^{1.3}$	52° C. for 1 hour	38	14	<100
	Distilled Water	52° C. for 1 hour	21	8	<100
	Citrate-Borate	52° C. for 1 hour	86	<2	8,000
Non-dialysed MEL	Citrate-Borate	52° C. for 1 hour	160	20	200
	Citrate-Borate	56° C. for 30 minutes	160	<2	8,000

It can be seen that it was not possible to convert the virus to a full indicator in these concentrations of calcium, although the enzymic activity was destroyed.

Dialysed MEL was converted to the indicator state by standard treatment at 52° C. Conversion can occur at temperatures as low as 40° C. for 30 minutes (Burnet, 1952). Non-dialysed MEL in citrate-borate must be heated at 55–56° C., but Stone has shown that it may become an indicator after heating at 37° C. for two hours in the presence of calgon.

DISCUSSION.

The present results define rather clearly the conditions under which a typical influenza A virus strain is or is not converted into the indicator phase by heating at 56° C. We find ourselves, however, at a loss to make any suggestions as to the mechanisms involved. Presumably we are concerned with a process of limited denaturation by heat of certain of the protein components of the virus particle surface. The specific biological reactions by which the process is followed are, however, much more delicate than any of the methods used in orthodox studies of protein denaturation and the virus particle itself is certainly much more complex than any pure protein. Reported studies of the influence of salts on denaturation of proteins by heat (review by Neurath

et al., 1944) are concerned with much higher concentrations than are used in our experiments and no useful analogies have been recognized. Lauffer and his colleagues (Lauffer and Carnelly, 1945; Lauffer and Scott, 1946; Scott and Lauffer, 1946) studied the thermal destruction of PR8 haemagglutinin in buffers of constant ionic strength but they made no observation on the indicator state of the virus.

The point of chief interest to us has been in regard to the negative correlation of enzymic activity and the indicator state. An examination of Figs. 5, 6, 7, and 8 will show that with citrate and calgon there is a precise change-over within a narrow range of salt concentration from indicator with no enzymic activity, to normal virus with full enzymic activity. The ranges concerned in these two cases are very dilute $M/10^{3.0}$ for citrate, $M/10^{4.6}$ for calgon. With sodium chloride we find with increasing concentration of salt a phase in which partial indicator activity develops before the enzyme is destroyed. All enzymic activity, however, disappears as the full indicator titre develops. With sodium sulphate the picture is similar.

There is still no adequate interpretation of the significance of the indicator state. The striking finding that with simple heating of LEE (Burnet, 1948) or heating of MEL in sodium citrate or calgon solutions (Stone, 1949, and present paper) there is a strict inverse correlation between enzymic activity and the indicator character, points strongly to the likelihood that the possession of active enzyme in some way prevents the effective adsorption of virus to inhibitor that is a necessary feature of indicator virus. There are undoubted examples where virus damaged in one way or another shows in the standard tests as an indicator or partial indicator and as having enzymic activity against the same inhibitor. The intermediate zone with sodium sulphate in Fig. 6 represents such a situation. However, when an enzyme is brought to a state of partial destruction, it must be expected that multiple tests for its activity will not necessarily give the same relative values as are obtained with undamaged virus. The intermediate zones shown in Figs. 5 and 6 do not eliminate the possibility that the indicator property of a virus preparation is wholly a reflection of the state of the enzymic units on the virus particle.

On this view the conversion of virus to indicator occurs whenever a process of denaturation can be so controlled as to destroy the capacity of the virus enzyme to activate and destroy its substrate yet produce no more than minimal damage to the capacity of the enzyme for specific adsorption to its substrate. This seems the simplest admissible working hypothesis. We are aware of the possibility that the correlation may be only an indirect one. It may be that the present results may offer some suggestions as to how such a process of controlled denaturation might be applied to more orthodox enzyme systems to produce modified enzyme which can combine specifically with its substrate without disrupting it.

SUMMARY.

A study has been made of the ionic conditions under which an influenza A strain, MEL, is converted to the indicator state.

Dialysed virus, like crude allantoic fluid virus, was not converted to indicator by simple heating in the pH range 7–9. However, the virus became an indicator after heating at 56° C. for 30 minutes at pH 10.

At pH 6·9, salts protected the haemagglutinin of dialysed virus against inactivation by heating. The dilution to which the salts protected against thermal destruction was a function of the valency of the anion, the activity increasing in the order (for sodium salts) chloride, nitrate; sulphate, oxalate; citrate; hexametaphosphate.

Around pH 8, dialysed virus heated at 56° C. lost about 50 p.c. of its haemagglutinin titre. With increasing concentration of various salts there were successively further destruction of haemagglutinin with incomplete change to indicator and then protection of haemagglutinin titre and conversion to the full indicator state.

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THE MECHANISM OF THE TRANSMISSION OF MYXOMATOSIS IN THE EUROPEAN RABBIT (*ORYCTOLAGUS CUNICULUS*) BY THE MOSQUITO *AEDES AEGYPTI*

by FRANK FENNER[1], M. F. DAY[2] AND GWENDOLYN M. WOODROOFE[1]

(From the [1]Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra; and [2]the Division of Entomology, Commonwealth Scientific and Industrial Research Organization, Canberra).

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The dramatic and widespread epizootic of myxomatosis amongst wild rabbits (*Oryctolagus cuniculus* (*L*)) in south-eastern Australia in the first three months of 1951 has focussed attention on the mode of transmission of the disease. Field evidence suggested that, although other insect vectors were of importance in certain areas and at certain times, it was highly likely that the explosive outbreaks which marked this epizootic were due to mosquito transmission (Ratcliffe *et al.*, 1952).

Several previous investigators have shown that mosquitoes can convey myxomatosis. Torres (1936) showed that *Culex fatigans* (Wied.) was able to transmit the disease. Aragão (1943) in a study of major importance for the elucidation of the natural history of the disease in America, showed that both *Aedes scapularis* (Rond.) and *Aedes aegypti* (*L*) could transmit myxomatosis from one Brazilian wild rabbit (*Sylvilagus minensis* Thom.) to another, or to a European rabbit (*Oryctolagus cuniculus*). Infections were produced at intervals up to 17 days after the infective bite, transmission being, in Aragão's view, mechanical in nature.

Independently of Aragão, Bull and Mules in Australia carried out investigations with several different species of mosquitoes. A preliminary communication only (Bull and Mules, 1944) has been published. They reported that *Aedes aegypti* readily transmitted the virus immediately after a feed on an infected domestic rabbit and for about 14 days subsequently. Transmission was effected by mosquitoes of each of the four species adequately studied.

In the present study investigations were carried out upon the mechanism of the transmission of myxomatosis by *Aedes aegypti*. No attempt was made in these experiments to simulate natural conditions of transmission as *Aedes aegypti* is not concerned with this in Australia. Instead answers were sought to two questions (1) is a "biological cycle" involved in mosquito transmission,

or is it entirely mechanical? (2) is the virus in the blood stream of the rabbit important from the point of view of mosquito transmission, as Houlihan and Lawson (1945) and a number of other authors have suggested?

Materials and Methods.

The strain of myxoma virus used was the same as that employed to initiate the epizootic in Australia, and was derived by repeated passage in laboratory rabbits from the strain originally sent to Rivers in U.S.A. by Moses (1911). It had been sent to Martin in England by Shope in 1934, and was brought from there to Australia by Bull in 1936 (Bull and Mules, 1944).

Wild rabbits (*Oryctolagus cuniculus*) were caught in the field in an area free of myxomatosis and kept singly in small cages for the duration of the experiments. Rabbits used for testing the infectivity of the mosquitoes by injection or by biting were destroyed when the disease became generalized, as no further readings could then be made upon them. This probably contributed to the successful avoidance of cross-infection. *Aedes aegypti* were bred in an insectary maintained at 80° F. and with a relative humidity of 60–80 p.c.

The flanks of the rabbits were depilated by plucking and subsequent clipping. Individual mosquitoes in glass vials were induced to feed through mosquito netting and the sites of the bites subsequently marked.

Titrations of blood were carried out by bleeding from the ear vein into citrate saline, and then making tenfold dilutions of this blood in normal saline containing 10 p.c. horse serum. Doses of 0·1 ml. of each dilution were inoculated intradermally in marked sites.

For the titration of mosquitoes, the latter were dissected into head plus proboscis, and the remainder of the body, and each part pooled and ground in small grinding tubes, without the addition of sand. The ground material was taken up in 0·25 ml. of normal saline containing 10 p.c. horse serum, 500 units of penicillin per ml., and 4 mg. streptomycin per ml., and tenfold dilutions were made in the same diluent. Doses of 0·1 ml. were inoculated.

Purely mechanical transfer was investigated by using stainless steel pins 0·01 inches diameter and projecting about 2 mm. from corks, each of which was inserted into a vial of the appropriate size. With these, the infected rabbits were pricked at the selected sites and healthy rabbits were pricked at marked sites at various intervals afterwards.

All inoculation sites were carefully observed from the third day until about the ninth day, if the animal survived as long as that. Local reactions to the mosquito bites or the pin pricks usually appeared between the fifth and the eighth day and always increased in size during the days following their appearance. Reactions at the sites of inoculation of material used for titrations were sometimes a little earlier, depending upon the dose.

There were always a number of test inoculations, bites, or pinpricks on each rabbit. Parker (1940) and Houlihan and Lawson (1945) showed that there was no interference between the reactions at such sites if the doses were all of the same general order of size. In the present series of experiments it was only in a very occasional case that any difficulty was experienced in deciding whether or not a reaction at a given skin site was a primary lesion due to inoculation in that site and not a secondary lesion due to localization of the virus in the inoculation site after its generalization through the blood stream. When secondary lesions did appear on the depilated skin, they showed no tendency to occur in sites of inoculation or mosquito bite more than elsewhere on the depilated skin, and as in mousepox (Fenner, 1948) the clinical character of primary and secondary skin lesions was different.

Throughout the experiments the mosquitoes were kept in the insectary (temperature 80° F. and relative humidity about 60–80 p.c.) and the pins out of direct sunlight in the laboratory (temperature about 70° F.).

EXPERIMENTAL RESULTS.

Preliminary experiments were based on the dissection and titration of mosquitoes which had fed (a) on the lesion caused by the inoculation of a large dose of virus, and (b) on a site distant from that lesion, at various stages of the disease. They showed that virus was present in the abdomen of mosquitoes which had fed on either site at any time from the third day after inoculation until the death of the rabbit. Positive results with ground head and mouthparts, however, were obtained only with mosquitoes which had fed on the primary lesion, and only in those which had fed on the lesion at a late stage of the disease.

A rabbit was inoculated intradermally, in a site from which the hair had been plucked, with 0·25 ml. of a 1/10 dilution of a suspension of freezedried virus, which contained about 10^5 egg infective ($= 10^6$ rabbit infective) doses per ml. A local lesion was apparent at the site of inoculation four days later, and on the seventh day there were early signs of generalization of the disease (blepharitis). Generalization was advanced on the eighth day and the animal died on the tenth day.

On the sixth day after infection the rabbit was encased in an unbleached calico bag, through which only the ears projected, and placed in a large cage containing several hundred recently emerged *Aedes aegypti*. 176 of these obtained a blood feed, and the engorged mosquitoes were then picked out and stored in the insectary in a gauze cage (20 in. × 20 in. × 12 in.) or singly in small vials or pill boxes. Resting mosquitoes were fed with sugar and water, and apple, and given the opportunity to take a blood meal from a guinea-pig on the second, sixth, and sixteenth days from the start of the experiment.

At daily intervals after the feed on the infected rabbit several insects were induced to feed at marked spots on normal rabbits. Two of these mosquitoes, which had never previously fed on a normal rabbit, were then anaesthetized with carbon dioxide. The head plus mouth parts were dissected from the rest of the insect, and the two portions ground in small mortars and inoculated in various dilutions on the other flank of the same rabbit. At the time of the initial biting of the infected rabbit, blood was obtained from an ear vein and titrated to determine the concentration of virus. The blood contained about fifty thousand infective particles per ml. of whole blood. About thirty stainless steel pins were used to puncture the skin of the ear in order to provide a mechanical analogy to the proboscis of a mosquito. At daily intervals one of these pins was inserted into a marked area of skin on the test rabbit, and discarded. Other pins were repeatedly used at intervals of 1, 2, 4, and 6 days.

Infection by interrupted feeding was investigated by inducing three mosquitoes to commence feeding upon the ears of the infected rabbit, and then complete their feed on the flank of a normal rabbit.

TABLE 1.

The results of attempts to transmit myxomatosis by mosquito bite and pinprick, using the ear of a rabbit, inoculated in the flank six days earlier as the source of the virus.

Interval—"infective feed" to day of test	Titration of mosquitoes		Feeding of mosquitoes	Pinpricks
	Head and Proboscis	Body		
0 (interrupted feeds)	0	1,000†	0/3*	0/4*
1	0	500	0/2	0/2
2	0	500	0/2	0/3
3	0	0	0/5	0/2
4	0	0	0/4	0/4
5	0	0	0/3	0/2
6	0	0	0/5	0/4
7	0	0	0/3	0/2
8	0	0	0/4	0/4
9	0	0	0/3	0/2
10	0	0	0/5	0/3
11	0	0	0/4	0/2
12	0	0	0/5	0/5
13	0	0	0/3	0/2
14	0	0	0/6	0/3
15	0	0	0/6	0/2
16	0	0	0/6	0/4
17	0	0	0/4	0/2
19	0	0	0/3	0/2
21	0	100	0/2	0/2
23	0	0	0/5	0/2
25	0	0	0/3	0/2
27	0	0	0/6	0/2
29	0	0	0/9	0/2

* numerator = number positive. denominator = number tested. † titre represented as number of infective particles per ml. of the original suspension of ground-up mosquito tissues.

The results of this experiment are set out in Table 1. It is apparent that mosquitoes which bit through tissues not infected with the virus, and imbibed blood containing virus, failed to transfer the infection by subsequently biting healthy rabbits. No positive results were obtained from 101 mosquito bites, made at intervals of a few minutes to 29 days after the infective feed. The mechanical analogy to the mosquito's proboscis, the steel pin, was likewise negative in all of the 64 tests made. The results of inoculations showed that in this experiment, as in three similar preliminary experiments, no virus could be demonstrated in the head and mouthparts. Virus could be demonstrated in the body for two days after the infective feed, and on the twenty-first day. The latter isolated positive result is analogous to the several positive results obtained with titrations of bodies of mosquitoes in the second experiment (Table 2).

Two days later, i.e. on the eighth day after inoculation of the infected rabbit, a similar series of operations was carried out, but on this occasion the mosquitoes were allowed to bite only on the site of the skin lesion resulting from the inoculation, and the pins were used in the same area. 108 engorged

mosquitoes were collected after an hour's exposure of the lesion. They were kept in a gauze cage in the insectary and were fed on sugar and water and apples. On the fourth and fourteenth days from the start of the experiment the resting mosquitoes were given the opportunity to feed on a guinea-pig. The testing of pins and mosquitoes followed the same plan as was used for the previous experiment. On this occasion the titre of the virus in the blood was about 10,000 infective particles per ml. of whole blood. The results of this experiment are shown in Table 2, and are in marked contrast to those just described.

TABLE 2.

The results of attempts to transmit myxomatosis by mosquito bite and pinprick, using the local skin lesion of a rabbit inoculated in the flank eight days earlier as the source of the virus.

Interval "infected feed" to day of test	Titration of Mosquitoes		Feeding of Mosquitoes	Pinpricks				
	Head and Proboscis	Body		day	Pin used every second day	Pin used every fourth day	Pin used every sixth day	Pin used test day only
0 (interrupted feeds)	10[1]	10,000[1]	3/5*(4,4,6)†	+(5)	0	+(7)	+(7)	—
1	0	100	0/2	+(4)				0
2	0	0	0/4	+(5)	0			0
3	0	0	0/3	+(5)				0
4	0	0	1/4(5)	+(5)	0	0		+(5)
5	0	0	0/3	0				0
6	10	10	0/2	0	0		+(9)	0
7	0	0	1/4(8)	+(7)				0
8	0	10	3/6(5,5,6)	0	0	0		+(5)
9	100	100	2/3(5,5)	0				0
10	10	100	1/4(7)	0	0			0
11	0	0	1/5(8)	0				0
12	0	0	2/5(6,7)	0	0	0	+(7)	0
13	10	10	1/4(7)	0				0
14	0	0	0/5	0				0
15	100	100	0/4	0				0
17	100	0	0/3	0				0
19	0	0	0/2	0				0
21	0	10	0/4	0				0
23	10	0	0/6					
25	0	0	1/5(7)					
27	0	0	0/3					

* numerator = number positive. denominator = number tested.

† (4) etc. = interval in days from test to first appearance of local lesion.

[1] Titre represented as the number of infective particles per ml. of the original suspension of ground-up mosquito tissues.

Infection by bites and pins was obtained on a number of occasions. Interrupted feeding, or immediate transfer of pins, was positive in the majority of tests. A proportion of positive results was obtained in most tests made up to the thirteenth day, and in a few tests subsequently, up to the twenty-fifth day. In all 16 out of 72 bites were positive, and 12 out of 53 tests with pins.

The results of biting experiments, and the inoculation of the ground mosquitoes, failed to agree on numerous occasions. Positive results were obtained at the site of mosquito bites, and negative inoculations of the head and mouth parts of the same mosquitoes, on the 4th, 7th, 11th, 12th, and 25th days, whereas the reverse held on the 6th, 15th, 17th, and 23rd days. Positive results by both methods of test were obtained upon only four occasions.

The two experiments are mutually confirmatory. Virus which passes to the gut of the mosquito (in the infected blood) appears to play no part in the transfer of the disease. When, however, the mosquitoes had to probe through the infected tissue of the local lesion to obtain their blood meal the proboscis of a proportion of the feeding mosquitoes became contaminated with virus, just as did a proportion of the pins stuck into the lesion. Infection was transferred when these contaminated probosces or pins were stuck into the skin of a normal rabbit, but even then infection did not invariably occur, as shown by the results with the pin tested each day. This produced a lesion each day until the fifth day, and then after two negative results again produced a lesion on the seventh day.

The next experiments were designed to determine, if possible, the relative infectiousness of an inoculated rabbit at different stages of the disease, if mosquitoes bit through the local lesion, and to obtain more data upon the ability of mosquitoes to transmit the disease upon more than one occasion by repeatedly biting normal rabbits. First, a rabbit was inoculated subcutaneously and intradermally with the virus. A well developed lesion was present on the fourth day and the animal died on the eighth day. Numbers of mosquitoes were fed upon the lesion and upon an area of the body remote from the site of inoculation each day from the fourth day until the rabbit died. Although there was an unfortunate lack of test bites on the seventh and eighth days, due to death of a test rabbit the day after it had received several bites, positive results appeared to be more frequent in the later stages than in the earlier stages of the disease. As in the previous experiments, mosquitoes feeding on a skin site distant from the lesion failed to convey the disease.

TABLE 3.

Results of feeding mosquitoes on the lesion, and on a body site distant from the lesion, of a rabbit at daily intervals after the first appearance of the lesion.

Day of disease	Fed on lesion	Fed on body distant from lesion
4	0/4	0/2
5	1/8* (8)†	0/5
6	4/7 (6,7,7,8)	0/8
7	0/3	0/7
8	1/1 (7)	0/2

*† symbols as in Table 2.

However, a positive result was obtained from the inoculation of a pool of the heads and mouthparts of six mosquitoes which had fed on a skin site distant from the primary lesion on the seventh day of the disease. This indicates that there was at least one potentially infective mosquito in the batch, not an unexpected result at this late stage of the disease, when secondary localization in the skin was occurring.

Next, two animals were inoculated intradermally in three areas on the back. There were well developed lesions on each on the fifth day. One rabbit was used daily from the fifth to the eighth day. On the ninth day this animal was dead, so the other one was used. Next day it also was dead. Although it was quite cold, twelve mosquitoes were induced to probe the lesions repeatedly, and were subsequently tested by interrupted feeding, as well as by biting several days later.

Several mosquitoes were induced to feed each day and they were kept singly in numbered vials. Each day thereafter until they died all the mosquitoes were given the opportunity to feed on the depilated skin of normal rabbits; when feeding occurred the site was marked and observed regularly to determine whether a local lesion developed there. Several important facts emerged from this experiment, and the results have been set out in some detail in Table 4. It is apparent that there is a steady decrease in the proportion of infecting bites with the passage of time after the infective feed, and the results of this experiment show conclusively that the "negative period" shown in Table 2 (first, second and third days) was due to the operation of chance, when small numbers of mosquitoes were involved. There is no latent period, but a progressive decrease in the effectiveness of biting with the passage of time. Chance again plays a rôle in determining whether a particular bite, by a mosquito which may harbour the virus on the proboscis, is infective. Occasionally (e.g. mosquito number 80) one mosquito repeatedly caused infection by successive bites. Frequently the first bite only was infective and subsequent ones negative, sometimes (e.g. mosquitoes 66, 73) the first bite was negative and a later bite positive, and occasionally (e.g. mosquito numbers 30, 43) one or more negative bites intervened between two positives. These experiments help to explain the lack of correlation between the results of feeding experiments and titrations observed earlier. The results obtained with mosquito 43, in which six negative bites were recorded between a positive on the first day, and a series of positives on the tenth, twelfth and fourteenth days, and mosquito 82, where there was a similar gap from the interrupted feed until the ninth day, are hard to explain. Taken alone, they might well be regarded as evidence for the existence of a latent period of the virus in the mosquito. However, the overall distribution of positive bites, calculated on the basis of the interval between the infective feed and subsequent feed (Table 4) shows conclusively that there is no latent period.

TABLE 4.

The results of repeated feeding on normal rabbits by mosquitoes which had bitten through the skin lesion of an infected rabbit at various stages of the disease.

Mosquito Number	Infective Feed: Rabbit	Infective Feed: Day of disease	Interval (days) between Infective Feed and subsequent Feeds. 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Percentage of Mosquitoes Demonstrably Infective.
1				..*	+(4)*	†*															
2				..	—	..	—		—	—		—	—	..	—	—		†			
3				..	—	..	†														
4	300	5		..	—	..	—	—	..	..		†									38
5				..	—	..	..	—	—	..		†									
6				..	—	..	+(7)	—	—	..		—	..	—	—	..	—		..	†	
7				..	—	..	—	—	..	..		†									
8				..	—	..	+(7)	..	—	..		†									
10				+(4)	..	—	†														
12				—	..	..	..	—	..		—	..	—	..	—	..		—	..	—	
13				+(4)	+(3)	..	..	—	..		†										
14	300	6		..	+(3)	..	..	†													63
15				..	+(3)	—	—	—	—		—	—	—	..	..	†					
16				..	—	..	..	—	—		—	†									
17				..	—	..	..	†													
18				..	+(3)	..	..	..	—		—	..	—	—	—	—		—	..	—	
21				..	..	+(6)	—	..		†											
22				..	+(5)	..	—	..		†											
23				..	..	+(8)	†														
24				+(3)	..	..	+(6)	..		†											
25				—	—	..	—	..		—	—	..	—	..	—		—	..	—		
26				..	..	—	..	..		†											
27				..	+(5)	—	..	—		—	..	..	—	—	..		..	..	—		
28				..	..	+(6)	..	—		—	..	—	..	—	—		†				
29	300	7		..	+(5)	..	—	..		†											71
30				..	..	+(6)	..	—		+(5)	..	—	..	—	..		—	†			
32				..	..	—	..	—		—	†										
33				..	—	..	†														
35				..	..	+(8)	†														
36				+(3)	..	—	..	+(4)		†											
37				+(3)	..	—	..	—		—	..	—	..	—	..		†				
38				.	..	—	†														
40				..	..	+(6)	—	..		†											

TABLE 4 (continued).

Mosquito Number	Infective Feed: Rabbit	Infective Feed: Day of disease	Interval (days) between Infective Feed and subsequent Feeds. 0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Percentage of Mosquitoes Demonstrably Infective.
43				+(5)	—	..	—		—	—	..	—	—	+(5)		+(8)	..	+(6)	
44				..	+(6)	—	..		—	..	—	—	—	—		—	..	—	
45				..	—	—	..		†										
46				+(7)	..	..	—		—	..	..	†							
47	300	8		..	—	..	..		†										60
48				+(7)	..	+(5)	..		†										
49				..	—	..	..		†										
51				..	..	+(5)	..		—	+(6)	—	..	—	—					
52				..	..	—	..		—	..	—	—	..	—		—	..	—	
53				..	..	+(5)	..		—	..	—	..	..	†					
57				..	—	..		—	—	..	—	—	..		—	..	—		
59				+(6)	..	..		†											
60				+(4)	..	—		+(5)	..	—	—	—	..		†				
61				..	+(5)	..		†											
64				..	—	..		—	†										
65				..	+(5)	†													
66	301	9		..	—	..		+(5)	—	..	—	..	..		†				71
67				..	+(6)	..		†											
68				—	..	..		†											
69				..	+(6)	..		—	+(6)	..	+(5)	—	—		—	..	—		
70				..	—	..		—	..	+(6)	—	..	—		†				
71				..	—	..		†											
72				..	—	..		+(5)	..	—	..	—	..		+(8)	..	..		
73				..	—	..		—	+(6)	..	..	—	..		—		—		
74			+(5)	+(6)	..		†												
75			..	..	—		—	—	..	..	—	..			—	†			
76			+(4)	..	—		—	—	..	..	—	—			—	..	—		
77			—	+(6)	..		†												
78	301	10	+(5)	..	+(4)		..	†											92
79	(dead)		+(5)	..	..		†												
80			+(5)	..	+(4)		+(5)	+(6)	+(6)	+(6)	—	..			†				
81			+(5)	+(7)	..		—	..	—	—	..	..			—	..	—		
82			+(5)	..	..		—	..	—	—	..	+(6)			—	..	+(6)		
83			+(5)	..	—		—	—	—	—	..	—			—	†			
84			+(5)	..	—		—	—	..	†									
85			—	+(7)	..		†												
Percentage of bites positive			82	82	37	43	19	17	16	25	6	6	0	10	20	20	14	0	

* + = local lesion at site of bite.
— = no lesion at site of bite.
.. = mosquito did not feed.
† = mosquito died on day indicated.
blank space = feeding not attempted.
(4) etc. = interval in days from bite to first appearance of local lesion.

In this experiment feeding was not commenced until there was a well developed lesion at the inoculation site. Nevertheless, the proportion of infected mosquitoes obtained by feeding on the lesion on the fifth day was considerably less than occurred subsequently.

As previous experiments had shown, attempted feeding on the lesion of a rabbit which has just died of myxomatosis is a most efficient method of rendering mosquitoes infective, if the insects are tested by allowing them to obtain a feed shortly afterwards on a normal rabbit. Two factors may be concerned. Mosquitoes placed on the lesion of a rabbit which has recently died have been observed to probe the skin repeatedly in an attempt to obtain blood, and the chance for puncturing virus-infected cells may therefore be considerably increased. On the other hand, the high proportion of positive results may be due to the fact that interrupted feeding of these mosquitoes was easily accomplished, as they had failed to obtain a blood meal from the dead rabbit. Thus the time-interval between contamination of the mouth parts and the opportunity to transfer the infection was reduced to a minimum. It is likely that both mechanisms operated in producing the high percentage of successful transfers observed at this stage.

The feeding and titration experiments showed clearly that infection is transferred by contaminated mouth parts, and the failure of mosquitoes to become infective if the only virus with which they had come in contact was that present in the blood was clearly demonstrated in the first experiment. Nevertheless, it was thought worthwhile to apply two other tests to determine the fate of virus ingested with the blood meal of a mosquito feeding through normal rabbit skin. First, twenty mosquitoes were fed on the primary skin lesions of a rabbit on the eighth day of the disease. Fifteen of them were kept in a clean gauze cage, removed twenty-four hours later, and dissected into head, proboscis, appendages, midgut, and the rest of the abdomen. Five mosquitoes of the same batch were also kept in small vials, and their excreta collected at the same time as the dissections were made. All samples were then titrated in normal rabbits, using one animal for the more concentrated suspensions and a second animal for the higher dilutions. Virus was recovered only from the pooled mosquitoes from which the excreta was derived, and, in high concentration, from the midgut. In other comparable experiments virus was recovered from the mouth parts, and from the excreta collected twenty-four hours after the infective feed, but not from that collected seven and forty-eight hours after feeding. The concentration of virus in the midgut was again high and there was no evidence of spread from the midgut to other portions of the mosquito.

Finally, an indirect test of the possibility that virus might pass from the gut to the haemocoele, and multiply in the mosquito, was made by testing for the possibility of transovarial transfer of the virus. Larvae and adults were

reared from the eggs of about 200 female mosquitoes, whose only blood meal had been from the ear or lesion of the infected rabbit used for the first two experiments. Twenty larvae were ground and inoculated into a rabbit, with negative results. Fifty adult female mosquitoes from the same batches of larvae failed to produce specific lesions when tested by inoculation, and the result of feeding twenty-six of these mosquitoes on normal rabbits was also negative.

DISCUSSION.

Two mechanisms exist whereby animal viruses can be transmitted by mosquitoes. The first and most common is that which occurs in yellow fever, dengue, and the viral encephalitides. In each of these diseases there is a considerable degree of host specificity, as far as the mosquito species is concerned; interrupted feeding either fails to transfer the disease (yellow fever, Philip, 1930; Kumm, 1932) or effects transfer only in exceptional cases (dengue, Simmons *et al.*, 1931); and there is usually a latent period of several days, sometimes called the extrinsic incubation period of the disease, between the infective feed and the development by the mosquito of the power to infect. In all of these diseases virus is imbibed with the blood, and the mosquito becomes infective only when a "biological cycle" has been completed, and virus has reached the salivary glands.

The second mechanism has been convincingly demonstrated, until the present experiments, only in fowlpox (Kligler and Ashner, 1929; Brody, 1936). Here there appears to be no specificity as far as the mosquito species is concerned, pins function almost as effectively as mosquitoes, interrupted feeding will transfer the virus, and there is no latent period. No careful experiments have been carried out with fowlpox to determine the relationship of viraemia to the infectivity of mosquitoes, but Brody (1936) reported that mosquitoes which fed on uninfected areas of the combs of diseased birds failed to transfer the disease while mosquitoes which fed on lesions were infective.

There seems little doubt from the experiments just described that the mode of transmission of myxomatosis is analogous to that of fowlpox. This is not unexpected, for there are good reasons for regarding myxoma virus as a member of the pox group of viruses, with specific rank equivalent to that of fowlpox and the mammalian pox viruses (Fenner, 1952).

Several significant points follow from the experiments reported here. Mosquitoes fail to become infective unless they contaminate the proboscis by penetrating infected epithelial cells. The demonstration, by inoculation, of virus in the bodies of mosquitoes caught in the field, is therefore not a satisfactory method for determining their infectivity rate. A positive result on inoculation does not necessarily mean that the mosquito was infective. The best way to make such determinations is by inducing the captured mosquitoes to feed on a normal rabbit, as Aragão (1943) did. If this proves impossible as it may with

some species of mosquito, dissection of the mosquitoes into head and proboscis, and body, and the separate inoculation of suspensions of these parts, will yield more information than inoculation of the undissected mosquitoes.

This mode of transmission of myxoma virus by mosquitoes results also in infection being produced by intradermal inoculation rather than intravenous, as occurs in malaria, dengue, yellow fever and other diseases in which the virus is injected with the saliva into the lumen of a small vessel. The mechanism of infection in myxomatosis may therefore be expected to conform, in its broad outlines, with that encountered in mousepox (Fenner, 1948).

As far as mosquitoes are concerned, rabbits will not serve as sources of transmissible virus until multiplication in the skin has proceeded to some extent, and it is likely that they become much more effective sources as the disease progresses, and the skin lesions develop. By analogy with mousepox, virus may be present in the skin of infected rabbits in appreciable quantities some time before the appearance of a lesion, but maximum titres are probably not attained until the lesion becomes obvious. The important rabbits from the epidemiological point of view, therefore, are those in which numerous and extensive skin lesions occur. Such animals were exceptional in this experimental series, and have been very rarely encountered in experiments with laboratory rabbits during the last nine months. In the field, however, a proportion of rabbits with advanced myxomatosis and many skin tumours is always seen in any area in which spread is active (Fenner, personal observations; Ratcliffe, 1951).

The irregular results obtained with both pins stuck into the skin lesions and mosquitoes feeding on the skin lesion can best be explained by the patchy distribution of virus through the tissues. Similarly, missed infections between successful transfers by a single pin or mosquito can be explained by the failure of one puncture to dislodge an infective dose of virus from the pin or proboscis, and the success of a later puncture.

From the 74 positive bites recorded in the present paper it is possible to estimate the average length of the incubation period in myxomatosis transferred by mosquito bite (*Aedes aegypti*) to wild rabbits (*Oryctolagus cuniculus*). If the first appearance of a skin lesion is taken as the end of the incubation period, this is $5{\cdot}4 \pm 1{\cdot}3$ days. The first signs of generalized disease (blepharitis and conjunctivitis) were usually observed on the seventh day after infection, and obvious sickness, of the degree recognizable in the field as myxomatosis, about two days later, although a number of rabbits died before there had been time for severe generalized myxomatosis to become apparent. These intervals are about a day longer than usually encountered with laboratory rabbits. The rabbits do not become infectious for mosquitoes until the skin lesions have developed. Some of the mosquitoes which penetrate these skin lesions are capable of transmitting the disease to normal rabbits immediately after this

infective feed, and with decreasing certainty of success for at least twenty-five days thereafter.

If the data obtained with *Aedes aegypti* can be used as a basis for generalization on the behaviour of the disease in the field, the interval between two generations of the disease is probably ten to twelve days. This period comprises eight to ten days between infection and the development of generalized disease, which is both the highly infectious stage for the mosquito and the clinically recognizable stage in the field, and two days, which may be an average interval between an infective feed and the subsequent blood meal on a normal rabbit. It is worth recalling, however, that in fowlpox Kligler and Ashner (1929) found that the incubation period in the fowl after an infective bite by *Culex pipiens* was about five days, and with *Aedes aegypti* between eight and eleven days. Similar differences associated with the species of mosquito may occur in myxomatosis also, and would necessitate a recalculation of the interval between two generations of the disease.

SUMMARY.

Female *Aedes aegypti* mosquitoes, which fed on the ear of a domestic rabbit which was infected with myxoma virus six days earlier, ingested virus with the blood but failed completely to transfer the disease, whether tested within a few minutes or at any time up to 29 days after the infective feed. Pins stuck into the ear of the same rabbit at the same time also failed to transfer the infection.

Female *Aedes aegypti* mosquitoes, which fed on the skin lesion of a domestic rabbit which was infected with myxoma virus eight days earlier, ingested virus with the blood and some also contaminated their probosces with virus. Positive transfers were obtained at intervals varying from a few minutes (interrupted feeding) up to 25 days after the infective feed. Pins stuck into the same skin lesion transferred the infection at intervals varying from a few minutes to twelve days.

The probability of mosquitoes becoming infected when probing through a skin lesion increased with the age of the lesion, and with well developed lesions between 60 p.c. and 70 p.c. of the mosquitoes became infectious. The probability that a mosquito with a contaminated proboscis would successfully transfer the disease diminished as the interval between the infective feed and a subsequent feed increased. Negative results not infrequently intervened between two positives.

Titration of dissected mosquitoes provided no evidence for either a transfer of virus from the gut to the haemocoele, or for multiplication of the virus in the mosquito.

Eggs laid by females whose only blood meal was upon an infected rabbit produced larvae and adults neither of which were infective upon inoculation into or by feeding on rabbits.

Transmission of myxomatosis by the mosquito *Aedes aegypti* is thus purely mechanical in nature, and is due to contamination of the mouth parts of the mosquito with virus during its passage through the virus-infected skin of an infected rabbit. With respect to its ability to transmit myxomatosis, *Aedes aegypti* is essentially a flying pin.

Acknowledgments. We wish to thank D. McIntosh and P. Wells for the assistance in maintaining the rabbit colony.

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ON THE PRESENCE IN RABBIT SERUM OF THROMBOTONIN (THROMBOCYTIN OR SEROTONIN)

by M. RAND AND G. REID[1]

(From the Department of Physiology, University of Melbourne).

(Accepted for publication 17th December, 1951.)

The blood of the rabbit, compared with that of the dog, ox, cat or human, is known to be rich in histamine (Barsoum and Gaddum, 1935; Code, 1937; Code and Ing, 1937; Code and Jensen, 1940). The distribution of the blood histamine is disputed; while there is agreement that it arises from the granulocytes in most species, so far as the rabbit is concerned there is conflicting evidence, Code finding its origin in the granulocytes and later observers (Zon *et al.*, 1939; Minard, 1941; and Grana and Rocha e Silva, 1945) regarding the platelets as its source. It has also been reported (Code, 1937; Grana and Rocha e Silva, 1945) that histamine enters the serum of the rabbit during clotting.

In the serum of many species including man, dog, ox, horse, sheep, and cat, there is another muscle stimulating substance which is quite distinct from histamine. This substance which arises from blood platelets and for that reason was called thrombocytin (Reid, 1943) is vasoconstrictor in action, and evidence is accumulating that it plays an important part in haemostasis (*vide* Quick, 1950). This vasoconstrictor substance which has been isolated from ox serum by Rapport (1949) has also been named serotonin. Quick (1950) has recently suggested a compromise name—thrombotonin and it is this name which we shall use throughout this paper.

Now histamine itself is vasoconstrictor in the rabbit, and in view of the evidence that it arises from platelets and enters the serum during clotting, the question may be raised whether in this species it plays the physiological rôle which thrombotonin plays in others. It has been known, of course, for many years that rabbit serum (like other sera) stimulates the isolated ox carotid artery but much of this evidence, particularly that of the earlier observers, is open to the theoretical objection that histamine may contract such preparations. This paper presents evidence that there is a muscle-stimulating and vasoconstrictor substance other than histamine in rabbit serum, and indicates on pharmacological grounds that this is thrombotonin.

METHODS.

Blood was obtained from rabbits by cardiac puncture or by carotid cannulation after ether anaesthesia. The blood was allowed to clot and the serum separated by centrifugation

[1] Technical assistance was provided by means of a grant from the National Health and Medical Research Council.

or it was collected into paraffin or silicone-coated glass vessels containing heparin. From such heparinized blood, "cell-free" plasma was separated by high-speed centrifugation and in four experiments various fractions of the white cell layer were separated by differential centrifugation, and suspended in saline. Serum or suspensions of the formed elements were (a) brought to the boil and filtered or (b) extracted with an equal volume of trichloracetic acid, filtered, and the trichloracetic acid removed from the filtrate by ether.

Samples of serum were electro-dialysed as described by Minard (1941). The serum diluted with two or three times its volume of saline was placed in the centre chamber, and the anode and cathode chambers, separated from it by cellophane, were filled with distilled water. The anode and cathode were connected to a 230 volt D.C. supply with suitable resistances so arranged that the temperature of the dialyser never rose above 40° C. The centre compartment was continually stirred with a small electric stirrer and a fan was arranged to blow over the surface to assist cooling and help remove chlorine. The whole apparatus was placed in an ice and salt mixture. The dialysate in the cathode compartment was kept for testing; its pH was read at 2 minute intervals and kept adjusted to pH 7 by the addition of normal HCl. Dialysis was continued for 1 hour.

Samples of serum from 4 or 6 white rabbits were pooled and extracted for thrombotonin by the method of Rapport, Green and Page (1948) obtaining as the final product a nitrobarbiturate complex.

Test-objects.

With two exceptions these have previously been described (Reid and Rand, 1951). The mouse jejunum or the rabbit jejunum was suspended at 37° C. in Tyrode solution in an isolated organ bath, 10 ml. in volume.

RESULTS.

The action of serum, protein-free filtrates and dialysates.

The uterus of the virgin rat. When rabbit serum in a dose of 0·05 ml. or more is added to a bath containing the rat uterus, the preparation contracts after a latency of several seconds. With small or moderate contractions there is also an increase in the amplitude and frequency of the spontaneous rhythm. With maximal or near-maximal contractions, the rhythm may be reduced in amplitude. On washing out the bath, the uterus returns to the baseline in 30 to 120 seconds. These responses which resemble those already described for thrombotonin (Reid and Rand, 1951) and for tryptamine (Reid, 1951) are not influenced when heparin is added to the serum. Heparinized plasma has no action, an inhibitory action or a weak stimulating action; but its stimulating action is much less than that of the corresponding serum.

Serum which has been diluted with an equal volume of saline and boiled, or trichloracetic acid filtrates of serum have an action similar to that of serum. Fig. 1 (panel 3) shows the response to 0·25 ml. of rabbit serum at S, to 1·0 ml. of the corresponding heparin plasma at P, and to 0·22 ml. of boiled serum at Sb. Each addition to the bath was followed by washing out 90 sec. later.

It is interesting to note that an electrodialysate of serum has an action on the rat uterus similar to that of serum itself, for Minard (1941) describes the

use of electrodialysis and direct testing of the dialysate without further modification as a means of estimating histamine. These results show that serum, protein-free filtrates and dialysates contain a substance which (since it stimulates the rat uterus) cannot be histamine.

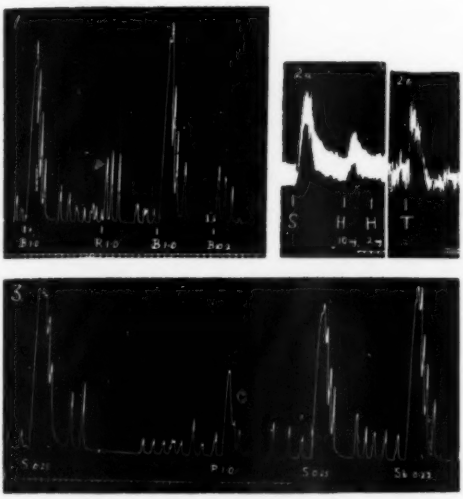

Fig. 1.

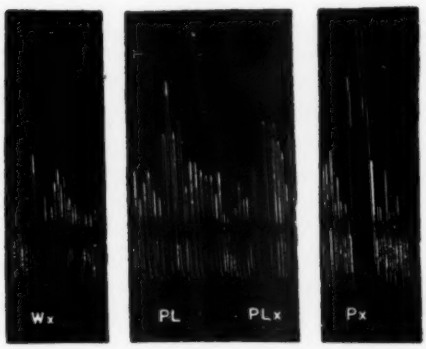

Fig. 2.

Fig. 1. Panels 1 and 3. Records of contraction of virgin rat uterus. At S 0·25 ml. of rabbit serum and at P 1·0 ml. of rabbit plasma were injected. At R an extract of red cells and at B an extract of the buffy coat were injected, the figures denoting mls.

Panels 2a and 2b. Records of contraction of jejunum of rabbit. Rabbit serum (0·4 ml.) was injected at S, histamine at H, and thrombotonin at T. Details in text. Time: half minutes.

Fig. 2. Record of rat uterus. An extract of the heavier elements of the buffy coat was injected at WX (0·06 ml.), an extract of the lighter elements was injected at PX (0·05 ml.). At PL and PLX respectively injections (1 ml.) of plasma and plasma extract were made.

The sheep carotid artery. Serum and protein free filtrates cause contraction of the spiral arterial strip after a brief latency. Plasma is inactive or has very much less activity than the corresponding serum. Sometimes this preparation responds to histamine in doses of 1 μg. or more but commonly as already described (Reid and Rand, 1951) it is insensitive even to 10 μg. of histamine.

The mouse jejunum. This preparation contracts after a latency of a few seconds to 0·05 ml. or more of rabbit serum, and is insensitive to 500 μg. of histamine. The magnitude of a maximal contraction is very much less than that of a similar length of guinea-pig jejunum. With repeated injections at

intervals of 5 minutes, the responses decline. Similar responses are given by tryptamine in doses ranging from 5 to 100 μg.

The rabbit jejunum. Rabbit serum in a dose of 0·1 ml. or more causes an initial inhibition of rhythm, followed in 5 to 10 seconds by a contraction during which the rhythm may be increased in amplitude (with small doses) or decreased in amplitude (with large doses). This preparation is relatively insensitive to histamine. For example, in one experiment, 2 μg. was without action and 10μg. caused a contraction very much less than that due to 0·2 ml. of rabbit serum. In other words, if the serum contraction had been due to histamine the serum must have contained more than 50 μg. per ml., which is out of the question. In the same experiment the addition of 10 μg. of benadryl abolished the action of 20 μg. of histamine but scarcely affected the action of serum. Panel 2a (Fig. 1) shows the effect of 0·4 ml. of rabbit serum at S, and of 10 μg. and 2 μg. of histamine respectively at H.

The guinea-pig jejunum. Since rabbit serum contains appreciable quantities of histamine the response of the guinea-pig gut may be predominantly due to histamine. Unfortunately the action of "histamine free" cat or human serum (and even of thrombotonin) is abolished by doses of benadryl little greater than those required to abolish the effect of histamine. However, the following evidence indicates that in rabbit serum there is a substance other than histamine which stimulates the guinea-pig jejunum.

(a) Recovery after benadryl: When histamine and filtrates of rabbit serum are added to the bath in alternate dosage each injection giving the same height of response the addition of benadryl (1–5 μg.) for 1 to 2 minutes abolishes the response to each. As the gut recovers after the benadryl is washed out the response to histamine recovers somewhat more slowly than that to serum or filtrate.

(b) When rabbit serum contains only a small amount of histamine (0·05 μg. per ml.) the response to untreated serum consists of a small immediate contraction followed in one or two seconds by a further response.

The action of extracts of the formed elements.

Panel 1 of Fig. 1 shows the action on the rat uterus of extracts of the red cells and of the "white cell" layer (or buffy coat) thrown down when the plasma used in the experiment of panel 3 was prepared. The cells were lysed by boiling in saline and the volume was made up to approximately the volume of blood from which they were derived. The red cell extract was injected at R and the extract of the buffy coat at B. Such experiments show that the activity is to be found in the buffy coat. In order to determine which fraction of it is responsible experiments of the following kind were performed.

A rabbit was injected with 500 units of heparin intravenously and 30 ml. of blood collected from the carotid artery into a chilled paraffin-coated centrifuge tube and centrifuged at 2,500 r.p.m. for 3 minutes. (International S.B.1 conical head). The supernatant plasma (16 ml.) was pipetted off. The small

amount of plasma remaining, together with the white cell layer was taken off and suspended in a few ml. of saline. The cloudy plasma was centrifuged at 3,000 r.p.m. for 45 minutes, the clear plasma removed and the deposit suspended in saline. By this means two suspensions were obtained, the first of those white cells thrown down early, and the second, of those white cells thrown down late. Trichloracetic acid extracts were made from each of the suspensions and from the plasma and tested on the rat uterus. The results are illustrated in Fig. 2. The doses of extract are expressed as though each had been made up to the volume of the original blood, viz. 30 ml. The extract of the heavier elements had a depressor action (at Wx) in a dose of 0·06 ml., the extract of the lighter suspension had a marked stimulating action (at Px) in a dose of 0·05 ml. The plasma extract (at PLx) had a slight depressing action in a dose of 1·0 ml.

The extraction of thrombotonin from rabbit serum.

The specificity of the method of extraction. The results so far clearly indicate that a substance other than histamine is present in rabbit's serum and they suggest that, as is the case with other sera, it has its origin in the lightest fraction of the formed elements of the blood. Attempts were made, therefore, to prepare thrombotonin from rabbit serum.

Before doing so it was necessary to examine the specificity of the method introduced by Rapport *et al.* (1948b) because hitherto its use has been confined to ox serum (Rapport *et al.*, 1948b; Reid and Rand, 1951). Ox serum contains so little histamine that the question of histamine appearing in the final nitrobarbiturate complex scarcely arises; this may not be the case with rabbit serum.

Ox serum, free or almost free from vasoconstrictor activity was prepared by recalcifying highly centrifuged plasma, as described previously (Rand and Reid, 1951). Two experiments were performed, each commencing with one litre of such serum to which was added histamine dichloride, in one experiment 15·2 mg. and in the other 17·2 mg. The serum was then extracted in the usual way, retaining some of the products for testing on the guinea-pig ileum, as indicated below:

1. The proteins were precipitated with ethanol, the filtrate concentrated, treated with acetone and again filtered. The "acetone precipitate" was kept for testing.

2. The filtrate was again concentrated and extracted with chloroform. The "chloroform layer" was taken to dryness and kept for testing and a fraction of the aqueous layer was also retained for testing after being freed of volatile organic solvents.

3. The bulk of the aqueous layer was adjusted to pH 5·6, saturated with ammonium sulphate and extracted with butanol. A solution of nitrobarbituric acid in methanol was added to the butanol layer and the precipitate kept for testing.

The results show that a fraction of the histamine (10–30 p.c.) appears in the "acetone precipitate." A considerable fraction (over 50 p.c.) remains in the aqueous phase up to the time of extraction with butanol, less than 0·1 p.c. appearing in the "chloroform layer." Nevertheless, no appreciable quantity of histamine appears in the final nitrobarbiturate complex. The nitrobarbiturate complex did have some slight depressor action and stimulating action on the guinea-pig gut; but so did the product from a blank run with serum to which no histamine had been added. In three further experiments, histamine (5 to 6 mg.) was added to 20 ml. of water and the pH adjusted to 4·5, 6·2 and 7·5 respectively. The solutions were saturated with ammonium sulphate and extracted with butanol. A nitrobarbiturate complex precipitated from the butanol layer contained, in each case, less than one-thousandth of the added histamine.

An experiment in which 11 mg. of l-adrenaline were added to a litre of inactive ox serum showed that about 80 p.c. persisted in the aqueous phase right up till the final stages but only about 1 p.c. appeared in the final product.

The identification of thrombotonin in rabbit serum. A nitro-barbiturate complex has been prepared from three batches of pooled serum ranging in volume from 140 to 200 ml. With such small volumes it is not possible, at present, to isolate and identify chemically the active substance in the same way as Rapport did with serotonin prepared from beef serum. We believe, however, that the presence of thrombotonin in such nitrobarbiturate complexes, and even in protein-free filtrates of serum, may be identified by its pharmacological actions. Many such pharmacological actions have already been described (Reid and Rand, 1951); practically we find it convenient to use the following :

1. Contraction of the carotid artery of the sheep, antagonized by yohimbine and potentiated by cocaine.
2. Contraction of the uterus of the virgin rat.
3. Contraction of the rabbit intestine, not affected by benadryl or atropine.
4. Contraction of the mouse jejunum.
5. Contraction of the guinea-pig jejunum, partially antagonized by benadryl and not affected by atropine.
6. Constriction of the pupil and retraction of the nictitating membrane on injection into a carotid artery of the atropinized decerebrate cat with divided cervical sympathetics and third nerves, the carotids being unligated.
7. Liberation of adrenaline from the suprarenal gland of the cat using the method of Feldberg and Minz (1931).

Since at present, we lack a sample of pure crystalline thrombotonin it cannot be said how much is necessary to elicit responses of these various test-objects; but we can say that amounts equivalent to 0·1 or 0·2 ml. of serum

give definite contractions of the first five, and definite responses of the last two are given by amounts equivalent to 0·5 or 1·0 ml. of serum.

The actions of thrombotonin on all these test objects, with the exception of the mouse and rabbit intestine, have been described before (Reid and Rand, 1951) and the actions of the nitrobarbiturate complex made from rabbit blood are qualitatively identical with them. Moreover, the ratio between equi-effective doses of the three different batches of nitrobarbiturate complex is of the same order for all the test-objects, further strengthening the view that we are dealing with the actions of a single substance.

The action of thrombotonin on the guinea-pig intestine especially when the period of contact of the drug is kept short (*circ.* 10 sec.) bears a superficial resemblance to that of histamine. With small doses the latency is appreciably longer than that of histamine. Fig. 3 shows the responses of a piece of guinea-pig jejunum to thrombotonin (prepared from rabbit serum) and to histamine. At the unlettered strokes 0·3 μg. of histamine was injected; at the arrows 1·5 mg. of nitrobarbiturate complex (representing the activity of 0·4 ml. of serum) was added. At the first down-pointing arrow 2 μg. of benadryl were added and a further 1μg. on washing out the bath after each of the next three succeeding injections. It can be seen that the response to thrombotonin is reduced but not to the same degree as that to histamine.

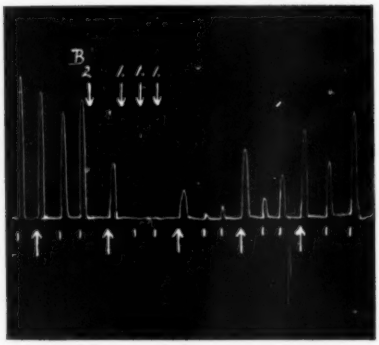

Fig. 3. Record of guinea-pig jejunum. At the up-pointing arrows, 1·5 mg. of thrombotonin and at the unlettered strokes 0·3μg. of histamine were given and left in contact for 15 sec. At the down-pointing arrows Benadryl in the doses indicated (representing μg.) was given.

The action of thrombotonin on the rabbit intestine is illustrated in panel 2b of Fig. 1. At T a nitrobarbiturate complex representing the activity of 1 ml. of serum was added. With partially purified thrombotonin the initial depression of rhythm (seen after serum itself) does not appear.

DISCUSSION.

Although it is well known that rabbit serum is rich in histamine, and there is some evidence (Zon *et al.*, 1939; Minard, 1941; Grana and Rocha e Silva, 1945) that its main source is the blood platelets, these results make it clear that serum and the lightest fraction of the formed elements contain yet another muscle-stimulating substance which can be extracted from serum and

on pharmacological grounds is identical with thrombotonin similarly prepared from ox serum.[2] The basis for this identification is similar in principle to the method of identifying acetylcholine in tissue extracts where chemical isolation is not possible (*vide* Dale, 1929). We consider that the reactions of the battery of test objects taken as a whole are characteristic of indolalkylamine derivatives. Apart from thrombotonin there is no known substance or conceivable mixture of substances derived from blood which gives this characteristic pattern of reactions.

It is clear, therefore, that histamine, although it is constrictor in the arterial tree of the rabbit, does not substitute for thrombotonin in that species. Indeed, it would seem highly improbable that a haemostatic mechanism of such general physiological importance should be mediated by a different agent in the rabbit from that in other animals. Moreover, in the rabbit histamine is a dilator of the smallest vessels causing hyperaemia when locally applied; when given intravenously the constrictor action is so prominent that it obscures the effect of the more peripheral dilatation (Feldberg, 1927). The function of the high histamine content of rabbit blood, however, remains unsolved. Is it possible that there is a synergism between it and thrombotonin? At present we have no information regarding the vascular actions of thrombotonin in the rabbit except that it constricts the vessels of the perfused ear (Rapport *et al.*, 1948); but experiments with the sheep carotid reported earlier (Reid and Rand, 1951) show that histamine frequently potentiates the action of serum vasoconstrictor.

It is worth emphasizing again how this substance may be mistaken for histamine in extracts of blood. This has been discussed in earlier papers (Reid, 1946; 1948) so far as sera of the horse, ox, dog, cat and man are concerned; but hitherto the problem of rabbit serum has been avoided because of its richness in histamine. In Minard's method of histamine extraction, an electrodialysate is used for the assay, and this we have shown to contain a muscle-stimulating substance other than histamine. Now since Code and Ing (1937) have chemically isolated histamine from the "white cell layer" of rabbit blood, there can be no doubt that histamine is present. But Minard, having shown that his electrodialysates have some physicochemical and pharmacological resemblances to histamine solutions, goes on to make the statement: "Since histamine has been isolated from the blood of the rabbit, it may be safely assumed that the active material is histamine itself." This argument does not prove that histamine is the only active substance present. Minard's dialysate does resemble histamine because there is little doubt that it contains histamine, but his pharmacological test-objects consisted only of the guinea-pig intestine and (in a few instances) the blood pressure of the cat; when tested on the rat

[2] Since this paper was prepared, Dr. M. M. Rapport has informed us that he has demonstrated serotonin by paper chromatography in platelet extracts from the rabbit and dog.

uterus it becomes clear that histamine is by no means the only substance present. Furthermore, when this other active substance is extracted from serum, it becomes apparent that it stimulates not only the rat uterus, but many other tissues, including the guinea-pig jejunum, the action on which when the period of contact is short, superficially resembles that of histamine, as Fig. 3 illustrates. These facts cast doubt on results of estimations of histamine in blood serum or plasma by methods which use the guinea-pig gut as the test object and do not get rid of, or avoid the possibility of the presence of, thrombotonin.

SUMMARY.

With Rapport's method for the partial purification of the vasoconstrictor of serum neither histamine nor adrenaline appear in the nitrobarbiturate complex in appreciable quantities.

Thrombotonin (thrombocytin or serotonin) has been isolated from rabbit serum in a partially purified form, and a method for its identification on pharmacological grounds is described.

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PORPHYRINS WITH FORMYL GROUPS II.[1]

PREPARATION OF CHLOROCRUOROPORPHYRIN AND DIFORMYL-DEUTEROPORPHYRIN

by R. LEMBERG AND J. PARKER[2]

(From the Institute of Medical Research, the Royal North Shore Hospital of Sydney).

(Accepted for publication 17th December, 1951.)

Chlorocruoroporphyrin, also called Spirographis porphyrin, forms the iron-free prosthetic group of chlorocruorin, the green blood pigment of some Sabellid worms. Its chemical structure is that of 1.3.5.8-tetramethyl-2-formyl-4-vinyl porphin 6.7-dipropionic acid or 2-formyl-4-vinyl-deuteroporphyrin IX (Fischer and v. Seemann, 1936). To this class of formylporphyrins also belongs the prosthetic group of the cytochromes *a* and cytochrome oxidase; this has been pointed out many years ago by Warburg and has been confirmed by recent work (cf. Lemberg and Falk, 1951). Moderately large amounts of pure chlorocruoroporphyrin were required for model studies on formylporphyrins, and for the study of its infrared spectrum (Falk and Willis, 1951). Sabellid worms are not readily obtained in large numbers. Fischer and Wecker (1942) synthesized chlorocruoroporphyrin by a complicated introduction of formyl and vinyl groups into deuterohaemin. Fischer and Deilmann (1944) obtained it by oxidation of protoporphyrin dimethyl ester with osmium tetroxide-hydrogen peroxide. None of these methods is, however, satisfactory for purposes of preparation.

The method of Fischer and Wecker gives extremely small yields. It requires numerous steps, including a laborious and difficult fractionation of 2- and 4-monoformyldeuteroporphyrin esters; in a later paper it will be shown that these two isomers had not been completely separated by the authors.

The osmium tetroxide-hydrogen peroxide process is described by the authors themselves as very tedious and requiring experience; osmium tetroxide is also an unpleasantly toxic reagent. The yield is given as 2·5–3 p.c., but the quantitative absorption data suggest that no pure chlorocruoroporphyrin was obtained. The absorption of chlorocruoroporphyrin has been correctly described by Stern and Molvig (1936) and has recently been confirmed by Lemberg and Falk (1951). The ratio of the extinction coefficients of the maxima

[1] See Lemberg and Falk (1951).

[2] This work has been supported by grants from the National Health and Medical Research Council of Australia.

of bands III and IV is 1·40, whereas the product of Fischer and Deilmann gave 1·11. As will be seen below, chlorocruoroporphyrin ester crystallizes together with 2.4-diformyldeuteroporphyrin ester, the latter having a ratio 0·60 of the two bands. In our hands the osmium tetroxide-hydrogen peroxide method gave erratic yields and had to be modified in order to give pure chlorocruoroporphyrin.

Fischer and Deilmann shortly described the oxidation of protoporphyrin ester with permanganate in acetone, with a yield of 1–2 p.c. of what was supposed to be almost pure chlorocruoroporphyrin ester. Our experiments described below leave little doubt that this was, in fact, an equimolar mixture of chlorocruoroporphyrin and diformyldeuteroporphyrin esters. The oxidation of protoporphyrin ester does not stop with the formation of chlorocruoroporphyrin ester. Simultaneously with the oxidation of the 2-vinyl group to formyl, a part of the second vinyl group is also attacked, leading to diformyldeuteroporphyrin ester; free carboxylic acid groups are also formed by further oxidation of formyl to carboxyl. It was therefore thought that the use of permanganate in greater dilution may decrease the oxidation beyond the stage of chlorocruoroporphyrin, or at least beyond that of formylporphyrins.

This has been achieved. An equimolar mixture of chlorocruoroporphyrin and diformyldeuteroporphyrin esters can be obtained in good yield. A chromatographic separation into the two components has been worked out, which though laborious gives the pure components with little loss.

A second purpose of this study was the investigation of by-products. It was hoped that some of the by-products might resemble porphyrin *a* from heart muscle more closely than does chlorocruoroporphyrin. Alslev and Kiese (1949) have obtained such a porphyrin in very poor yield by treatment of haemoglobin with nitrite and hydrogen peroxide, and removal of iron and protein from a choleglobin-like compound thus formed. This "verd$_{NO_2}$ porphyrin" had an oxorhodo-type spectrum (see Lemberg and Falk, 1951), though not as marked as porphyrin *a*, whereas chlorocruoroporphyrin has a rhodotype spectrum. Fischer and Deilmann had isolated from crude chlorocruoroporphyrin ester by an elaborate process of fractional alumina chromatography and crystallization an "isospirographis porphyrin" ester which had crystal shape and melting point similar to Kiese's porphyrin. They had reported that it had the same absorption spectrum as chlorocruoroporphyrin ester, but gave no data.

EXPERIMENTAL.

Methods.

Melting points were determined with a Griffin and Tatlock electric micro-melting-point apparatus with curved thermometer embedded in a copper block and are corrected. The melting could best be observed in reflected light, putting the crystals on a thin microscope coverslip covering the copper square over the hole through the copper block.

Absorption spectra were measured in the Hilger Uvispek spectrophotometer, and position of the bands was checked with the Hartridge reversion spectroscope. The position of the maxima found in the spectrophotometer agrees with the position of the band centres in the reversion spectroscope, except for band II which is skew and in the spectroscope appears to have a darker part towards the blue.

Chloroform was frequently used as a solvent, and it was found necessary to redistil it in all-glass apparatus. Chloroform for anaesthesia was found to contain sufficient zinc to form zinc complexes which appeared as green bands in the chromatograms. While zinc may be removed from porphyrin solutions in chloroform by shaking repeatedly with 25 p.c. (w./v.) hydrochloric acid, copper would not be thus removed. The complete removal of metal able to form complex salts is essential since the presence of even small amounts of complex would affect the absorption ratio of the bands.

The following sensitive check on all solvents is simple to carry out.

A trace of meso- or proto- porphyrin ester, sufficient to give a distinct four-banded absorption spectrum, is dissolved in about 10 ml. of the solvent. 0·1 ml. pure glacial acetic acid is added, the solution is evaporated on a water bath, and addition of fresh solvent and evaporation repeated thrice. After this, a solution in 10 ml. fresh solvent should show the unaltered aetio-type spectrum (bands increasing in strength from I to IV).

A relative increase of the strength of bands II and III indicates slight metal contamination; with a more heavily contaminated solvent, the four-banded spectrum is replaced by the two-banded one of the metal complex, and the position of the bands makes it possible to differentiate between zinc and copper.

Preparation of mixed crystals of chlorocruoroporphyrin and diformyldeuteroporphyrin dimethyl esters from protoporphyrin dimethyl ester.

100 mg. of protoporphyrin dimethyl ester (m.p. 230–232° C.) are dissolved in 400 ml. of acetone (A.R.) on a boiling water bath. A solution of 100 mg. finely powdered potassium permanganate (A.R.) in 50 ml. acetone is slowly run into the boiling acetone solution of the proto ester from a dropping funnel, in the course of 45 minutes. Manganese dioxide, occluding some porphyrin potassium salts (see by-products I, below) is removed by filtering the hot solution. After cooling, this is poured into 1 litre of peroxide-free ether. Acetone is removed by washing repeatedly with large volumes of distilled water, re-extracting the first extracts once again with ether. The collected ethereal solution is then shaken with 1 p.c. aqueous sodium carbonate which removes the free porphyrin acids (see by-products II, below). At this stage the absorption spectrum appears to be that of protoporphyrin, an absorption band at 555 mμ besides that of protoporphyrin at 538 mμ being the only evidence for the presence of formylporphyrins. Use of large amounts of permanganate makes the band I of the formylporphyrins at about 648 mμ appear besides that of protoporphyrin at 631 mμ, and increases the ratio of the band at about 555 mμ to that at 538 mμ; it does not, however, increase the yield of chlorocruoroporphyrin, while increasing that of diformyldeuteroporphyrin and of the free porphyrin acids. The ethereal solution is washed with water, dried over anhydrous sodium sulphate and evaporated to about 100 ml. On standing

overnight crusts of crystals are deposited on the vessel walls. The mother liquor contains the unaltered protoporphyrin ester, 25–35 mg. of which can be recovered from it, and only small amounts of formylporphyrin ester. The crystalline crusts still contain small amounts of proto ester which is removed by two recrystallizations from chloroform-ether. 15–20 mg. glittering leaflets of m.p. 282° C., beginning to sinter slightly at 272–274° C.

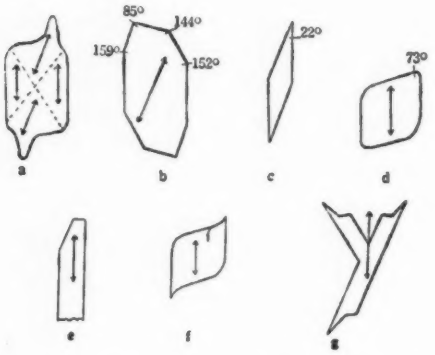

Fig. 1. Crystals of equimolar mixture of chlorocruoroporphyrin and diformyldeuteroporphyrin dimethyl esters. The double arrow represents E1, the vibration direction of polarized light which is maximally transmitted by the crsytal.

Most of the elongated plates of form a, Fig. 1, are interpenetration twins with strong pleochroism. The triangular segments on the straight longer edges of the plate have extinction and El parallel to these edges, while the segments on the shorter curved ends (which often carry protuberances) have El forming an angle of 20–28° with them; El being the vibration direction for polarized light which is least absorbed by the crystal. These crystals are occasionally accompanied by non-twinned rhomboid or six-sided plates (b–f, Fig. 1) and by twins of form g, Fig. 1, which resemble those of chlorocruoroporphyrin ester (Fig. 2, 6b). From benzene-ether diamond-shaped plates of form c, Fig. 1, are obtained.

Absorption spectra in the reversion spectroscope:

in chloroform: I 648·6, II 590·7, III 560·6, IV 520 mμ;
in ether: I 648·2, II 588·2, III 555·7, IV 516·4 mμ.

TABLE 1.

Absorption spectrum of crystals in comparison with that of chlorocruoroporphyrin and diformyldeuteroporphyrin esters.

Solvent: Chloroform.

	Position of maxima λ in mμ				Ratio of densities to that of band IV			
	I	II	III	IV	I	II	III	IV
Crystals	648	590	561	522	0·23	0·66	1·01	1
Chlorocruoroporphyrin	644·5	589	560	520	0·21	0·88	1·40	1
Diformyldeuteroporphyrin	651	595	562·5	526	0·28	0·51	0·61	1

The position of the band maxima, and the ratio of the extinction bands (Table 1) is intermediate between those found for chlorocruoroporphyrin and for diformyldeuteroporphyrin esters. The mother liquor of the crystals contains a slight excess of chlorocruoroporphyrin $R^{III}/_{IV} = 1{\cdot}07–1{\cdot}10$ and gives a further crop of crystals, rounded pleochroitic leaflets.

By-products and alteration of conditions of preparation.

On washing the manganese dioxide precipitate (by-product I, above) with water, potassium salts of porphyrins are extracted. Some of these porphyrins can be brought into ether by acidification with acetic or dilute hydrochloric acid (Ia). Absorption spectrum: I 640, II 588·5, III 553, IV 514 mμ in ether, aetiotype (IV> III> II>I) with rather strong band II. The remainder can be dissolved in chloroform (Ib) with greenish-brown colour. Absorption spectrum: I 648, II 593, III 559, IV 520 mμ (IV> II> III> I). None of these fractions gave crystalline material.

The free porphyrin acids (II, above) were brought into ether on acidification showing the following absorption spectrum: I 638, II 585, III 546, IV 509 mμ, aetiotype. Numerous attempts to isolate pure compounds from this mixture after esterification by HCl-fractionation, solvent fractionation and alumina chromatography led only to two crystalline materials, insufficient in amounts for further study:

IIa. 7–11 p.c. HCl fractions, ether-insoluble, eluted from alumina column by ether-chloroform (1:1). Rounded, strongly pleochroitic (dark brown-light grey) plates.

IIb. 9 p.c. HCl fraction, ether-soluble, eluted from alumina column by 80 p.c. ether-20 p.c. chloroform mixture. Clusters of needles.

All of these fractions had aetiotype spectra with similar bands varying between the following limits:

> I 636·5–643·5, II 584–591, III 550·5–557·0, IV 513·5–521 mμ, in chloroform. The lower HCl-fraction (4 p.c. and 8 p.c. HCl extractable) had distinct "glycol" bands at 608–610 mμ.

Attempts to separate chlorocruoroporphyrin ester from proto ester by Willstätter's HCl method gave poor results; after extraction of most of the proto ester by 4 p.c. HCl, the 8 p.c. HCl fraction, having been brought back into ether by neutralization, still showed absorption bands at 630 and 538 mμ, and the band at 630 mμ could still be observed in the neutralized 10 p.c. HCl fraction. The 8 p.c. HCl fraction contained mainly chlorocruoro, the 10 p.c. HCl fraction diformyldeuteroporphyrin ester, and after driving back into ether diformyldeuteroporphyrin ester crystallized from the latter fraction almost pure. It is essential to drive the porphyrin esters back into ether by neutralization with

sodium bicarbonate immediately after extraction with the hydrochloric acid in order to avoid saponification. There appears to be present another porphyrin with band I at about 630 mμ, but band III at 555 mμ. Such a porphyrin with band I at 625 mμ, III at 550 mμ in ether, was also observed in the 4 p.c. HCl fraction, after second permanganate oxidation of proto ester, recovered from the first oxidation and not specially purified. The Willstätter method was, therefore, used only to recover some additional chlorocruoroporphyrin ester from the mother liquors of the mixed crystals, and as first step of recovery of unaltered proto ester. A porphyrin with band I at 627 mμ could be separated from recovered proto ester by extraction of the ethereal solution with 0·5–1 p.c. HCl.

Addition of the permanganate solution to a more concentrated proto ester solution, or sudden instead of gradual addition, resulted in decreased yields of formylporphyrin esters and increase of free porphyrin acids. In addition, a compound with a strong absorption band at 668–671 mμ is formed, which remains in the mother liquors on crystallization of the formylporphyrin esters from chloroform-ether. Little of this compound is formed on slow addition of permanganate. Variation of the amount of permanganate between 70 and 140 mg. per 100 mg. proto ester had little effect on the yield of the formylporphyrin ester crystals. Sudden addition of 100 mg. permanganate following slow addition of 100 mg. gave a yield of 5 p.c. of almost pure diformyldeuteroporphyrin ester.

Chromatographic separation of chlorocruoroporphyrin and diformyldeuteroporphyrin dimethyl esters.

Attempts to separate the two compounds by fractional crystallization from chloroform-ether, benzene-ether, or chloroform-benzene, by adsorption chromatography of the benzene-ether solution on calcium carbonate, or of the ether and ether-chloroform solutions on aluminium oxide (U. S. Merck, standardized according to Brockmann), all failed. A number of other alumina preparations were equally ineffective. Aluminium oxide (B.D.H. for chromatographic adsorption "for preparative work") effected some separation, but filtration was extremely slow and soon failed to work. By mixing this alumina with more granular and less retentive alumina preparations (which themselves effected no separation) such as ignited alumina, Gen. Chem. Co., N.Y. or anhydrous alumina of Fisher, Eimer and Amend, moderately good separation was achieved. Finally, it was found that the "alumina for chromatographic separation" (B.D.H.) gave good separation, particularly if the column was introduced as a slurry in equal volumes of ether and chloroform, and the same mixture used for development. Apart from minimizing the risk of cavitation of the column, the addition of chloroform prevented the crystallization of the porphyrin esters in the column

provided the solutions were not too concentrated. A purple band of predominantly chlorocruoroporphyrin ester moves down the column far more rapidly than the brownish band of predominantly diformyldeuteroporphyrin ester. Impurities are retained on the top of the column and traces of protoporphyrin ester are eluted before the band of chlorocruoro ester.

The procedure was controlled by measuring in the various fractions the ratio of the absorption bands III and IV at their maxima (approximately 560 and 520 mμ) in the spectrophotometer in chloroform solution and uniting fractions of similar ratio. Owing to some tailing of the chlorocruoroporphyrin ester band, one fractionation does not give a complete separation. The ratio for the chlorocruoro fraction was about 1·30, that for the small middle fraction 0·70–0·80, and that for the diformyldeutero fraction 0·70–0·85. A second fractionation raises the first ratio to 1·34–1·38, and decreases the last to 0·63–0·70. After a third fraction the ratios reach their final values of 1·39–1·40 and 0·60–0·61, and further fractionation leaves these unaltered. The collected intermediate fraction of ratios 1·0–1·25 and 0·8–1·0 are then resubmitted to fractionation.

The chloroform solutions are evaporated to a small volume and crystallized by the addition of ether.

Chlorocruoroporphyrin dimethyl ester.

Thin prismatic crystals of m.p. 277–278° C. of various shapes (Fig. 2).

Calculated for $C_{35}H_{36}O_5N_4$ C: 70·91, H 6·13.
Found C: 71·00, H 6·49.

The absorption spectrum in neutral chloroform (Table 2) agrees well with that reported by Lemberg and Falk (1951) for chlorocruoroporphyrin ester from chlorocruorin. Absorption bands in the reversion spectroscope in 20 p.c. HCl: 615·2, 564·3 mμ (II, I); in chloroform shaken with 20 p.c. HCl: 621, (598), 573, (533·5) mμ.

TABLE 2.

Absorption spectrum of chlorocruoroporphyrin dimethyl ester in chloroform.

		Maxima					Minima			
		I	II	III	IV	V	I	II	III	IV
Chlorocruoro ester obtained by oxidation of proto ester	λ in mμ	644·5	585	560	520	421	627	575	539	473
	ϵ_{mM}	2·29	9·33	14·8	10·6	161	0·98	6·45	5·30	2·75
	ratio	0·217	0·885	1·40	1·0	15·2	0·093	0·61	0·50	0·26
Chlorocruoro ester natural (Lemberg and Falk, 1951)	λ in mμ	644	584	558·5	518·5	—				
	ratio	0·209	0·915	1·40	1·0	—				

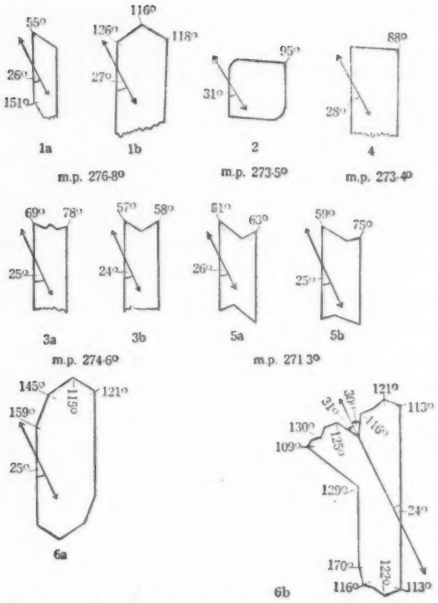

Fig. 2. Chlorocruoroporphyrin dimethyl ester.

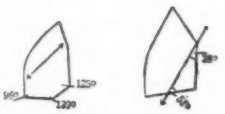

Fig. 3. Diformyldeuteroporphyrin dimethyl ester.

Crystal forms are given in Fig. 2; the numbers under the drawings represent fractions in the chromatographic separation from which the particular crystals were obtained. Most crystals are thin prisms or plates elongated along edge *d* which forms an angle of 25° with El (see above); occasionally the angle between dominant edge and El is found somewhat larger, 31° (e.g. in 2), and both edges may be observed in the same crystal (e.g. in 6b). The prisms end in a variety of crystal edges. The twins 6b, Fig. 2, greatly resemble the crystals obtained by synthesis from deuterohaemin and studied by Steinmetz, but the direction of extinction El was different. This difference is not due to the fact that Steinmetz studied his crystals in monochromatic yellow (mercury) light, and we in daylight, since it persisted when our crystals were studied in sodium light.

Chlorocruoroporphyrin dimethyl ester oxime.

Fine needles, m.p. 254–257° C. Absorption spectrum in the reversion spectroscope: I 636·3, Ia (607·5), II 584 (max. 579·5), III 541·5, IV 506·2 mμ in ether, aetiotype (cf. Lemberg and Falk, 1951).

Diformyldeuteroporphyrin dimethyl ester.

Strongly pleochroitic plates of irregular five or six-sided shape (Fig. 3). M.p. 301–303° C. Lemberg and Falk (1951) reported 280° C., Fischer and Deilmann (1944) 290° C. in melting point tubes, 303–305° C. in the Kofler micro-melting-point apparatus. The absorption spectrum (Table 3) differs little from that observed by Lemberg and Falk (1951). Absorption bands, in the reversion spectroscope, in chloroform shaken with 20 p.c. HCl: 624, (599), 576, (535), 444 mμ (in brackets weak bands).

TABLE 3.

Absorption spectrum of diformyldeuteroporphyrin dimethyl ester in chloroform.

	Maxima					Minima			
	I	II	III	IV	V	I	II	III	IV
λ in mμ	651	595	562·5	526	435	630	580	549	485
ϵ_{mM}	3·48	6·48	7·70	12·6	137·5	1·43	3·9	6·05	3·8
ratio	0·28	0·51	0·61	1·0	10·9	0·114	0·31	0·48	0·30

Preparation of chlorocruoroporphyrin with osmium tetroxide-hydrogen peroxide.

Fischer and Deilmann oxidized 100 mg. of protoporphyrin ester with osmium tetroxide-hydrogen peroxide, using an ethereal hydrogen peroxide solution containing 430 mg. H_2O_2 (74 moles H_2O_2 per mole porphyrin), and interrupted the oxidation after 15 minutes, when the protoporphyrin spectrum had practically disappeared. Diformyldeuteroporphyrin ester and only poor yields of chlorocruoroporphyrin ester were obtained. The results were better when the amount of hydrogen peroxide was halved (35 moles H_2O_2 per mole porphyrin) and the reaction interrupted after 12 minutes. At this time, much unaltered protoporphyrin was still present, which was removed as described above. On fractional chromatography a chlorocruoroporphyrin ester of m.p. 279–282° C. was obtained which had the absorption spectrum and crystal form described above.

Attempts to isolate "isospirographis porphyrin."

A careful chromatographic fractionation of chlorocruoroporphyrin ester obtained by the permanganate oxidation method and almost freed from diformyldeuteroporphyrin ester failed to reveal any significant deviation of the absorption spectra, except for the presence of a trace of protoporphyrin in the small first fraction, and of some diformyldeuteroporphyrin in the later

fractions. None of the fractions showed band ratios II/IV or III/IV higher than those of chlorocruoroporphyrin (Table 4).

Since Alslev and Kiese (1949) reported that their "verd $_{NO_2}$ porphyrin" ester, provisionally identified with "isospirographis porphyrin" ester, migrated in an alumina column together with protoporphyrin ester, whereas chlorocruoroporphyrin ester migrates after it, we have paid special attention to the small precursor fractions. "Isospirographis porphyrin ester" differs from chlorocruoroporphyrin ester according to Fischer and Deilmann in melting point and crystal form, isospirographis porphyrin ester having a lower m.p. (269–271° C.) and crystallizing in oval plates, not in prisms. If verd $_{NO_2}$ porphyrin were identical with isochlorocruoroporphyrin, the absorption spectra should also show a considerably higher ratio of absorption bands II/IV and III/IV (cf. Table 5) and a different band position.

The fractions of chlorocruoro ester obtained by permanganate oxidation showed considerable variation in crystal form, but only one (2, Fig. 2) deviated markedly from the others. The melting point of this fraction was, however, not lower than those of the other fractions. From various small precursor fractions protoporphyrin ester was removed by repeated crystallization from chloroform-ether. Three different preparations were obtained. The one, of m.p. 270–273° C., crystallized in diamond-shaped plates with slight re-entrant angle near the shorter diagonal (Fig. 4, a). The second, melting at 268° C. (incompletely) showed form b, Fig. 4, but on further recrystallization gave forms c and d (Fig. 4), which showed one edge which may be identical with edge *d* generally present in chlorocruoroporphyrin ester crystals. The third formed leaflets with rounded edges (e in Fig. 4). Table 5 shows that the absorption spectra of the forms 4 a and 4 e in chloroform deviated slightly from those of pure chlorocruoro ester in having somewhat higher bands I and II. The ratio of band III to IV was, however, not significantly different from that of chlorocruoro ester and the absorption spectra were still far closer to that of chlorocruoroporphyrin ester than to that of the Kiese porphyrin, both in position of the bands and their ratios. The amount of these precursor fractions was very small.

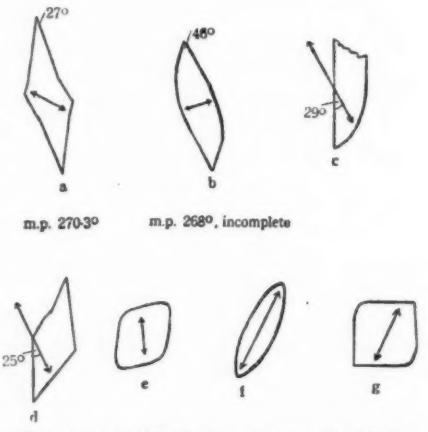

Fig. 4. Crystals from precursor fractions.

TABLE 4.

Chromatographic fractionation of almost pure chlorocruoroporphyrin ester.

Fraction	Position of absorption bands in reversion spectroscope (R, R'*) and of maxima of absorption in the spectrophotometer (H) λ in mμ									Ratio of maximal extinctions		
	I		II			III		IV		I/IV	II/IV	III/IV
	R	H	R	R'	H	R	H	R	H			
1	645·1(632)	645	588·2	583·8	584	560·0	560	517·7	518	0·216	0·884	1·30
2	644·9	644·5	590·1	584·5	585	560·3	560	519·7	520	0·220	0·876	1·34
3†	644·6	645·5	588·9	583·7	585	559·8	560	520·9	521	0·213	0·87	1·37
4†	644·3	645·5	589·5	584·3	585·5	560·4	560	521·5	521	0·213	0·87	1·38
5†	644·6	645	589·3	583·6	585	560·3	560·5	521·1	521	0·205	0·883	1·38
6	645·5	645·5	589·4	582·9	586	595·9	560	521·4	521	0·210	0·83	1·31
7	646·0	647	590·4	583·3	586	560·9	560·5	521·6	521	0·214	0·815	1·27
8	646·3	646·5	589·6	583·8	586	560·5	560·5	521·6	521·5	0·208	0·815	1·25
9	647·2	647	589·8	583·6	586·5	560·8	560·5	522·9	522	0·227	0·78	1·20
10	647·6	648	589·4	583·5	587·5	560·5	560·5	522·7	522·5	0·226	0·73	1·15

* Band II has a stronger part in the reversion spectroscope, the centre of which is given under R'.

† Main fractions.

TABLE 5.

Absorption spectra of precursor fractions in chloroform.

	Maxima λ in mμ				Absorption ratio		
	I	II	III	IV	I/IV	II/IV	III/IV
4 a	644·5	585	560	519·5	0·244	0·98	1·38
4 e	645	584·5	560	519·5	0·250	1·06	1·42
4 f, g	645	586	560	520	0·207	0·89	1·40
Chlorocruoro	644·5	585	560	520	0·217	0·88	1·40
$Verd_{NO_2}$	642	581	558	518·5	0·325	1·12	1·67

The osmium tetroxide-hydrogen peroxide preparation gave a precursor fraction (m.p. 260–264° C.) crystallizing in lance-shaped plates of form f in Fig. 4, accompanied by some almost square plates (g). The absorption spectrum of their chloroform solution (see Table V, 4 f, g) showed no significant deviation from that of the prismatic chlorocruoroporphyrin ester crystals, nor was there any deviation in position and strength of the minima. On successive recrystallizations from chloroform-ether, the melting point rose gradually to 274–276° C., and alumina chromatography finally gave typical prismatic crystals of m.p. 279–282° C.

DISCUSSION.

Data reported in this paper show that closely related porphyrins can form mixed crystals which simulate a chemical individual. The melting point is no safe criterion since the melting point of the equimolar mixture was not below that of chlorocruoroporphyrin ester, although somewhat earlier sintering could

be observed. Fractionation by alumina chromatography allowed separation of the closely related divinyl (proto), vinylformyl (chlorocruoro), and diformyl-deuteroporphyrin esters. It is not certain whether this method, or fractional crystallizations would separate isomers, such as 2-formyl-4-vinyl (chlorocruoro) and 2-vinyl-4-formyl deuteroporphyrin esters, although we have recently found that a separation of 2-formyl and 4-formyl deuteroporphyrins may be carried out by either method (unpublished). No definite evidence for the presence of 2-vinyl-4-formyldeuteroporphyrin ("isospirographis" or isochlorocruoroporphyrin) accompanying chlorocruoroporphyrin in our preparations was found, but the fact that the vinyl group in 4 is finally oxidized with formation of diformyldeuteroporphyrin and the variation of melting points and crystal forms makes this appear likely. If our and Fischer's preparation initially yield mixtures of chlorocruoroporphyrin and isochlorocruoroporphyrin, the latter must have an absorption spectrum virtually identical with that of chlorocruoroporphyrin, and it is therefore unlikely that Kiese's verd$_{NO_2}$ porphyrin is isochlorocruoroporphyrin. Further observations on verd$_{NO_2}$ porphyrin will be published in a separate paper.

The well-known Willstätter HCl-method meets with special difficulties with formylporphyrins, since their solubility in ether is small and greatly influenced by traces of impurities. An ideal column technique using the kationic rather than the anionic properties of porphyrins as basis of separation, is still missing, although the recently published study of Lucas and Orten (1951) provides the first approach to it. It is certainly not incidental that acid-washed aluminas were found effective in the separation of chlorocruoro and diformyl-deuteroporphyrin esters, while alkali-washed alumina preparations were quite ineffective. Paper chromatography has been introduced into porphyrin chemistry by Nicholas and Rimington (1949), but this method makes use of the anionic properties of porphyrins. It, therefore, separates well, porphyrins having a different number of carboxyls, but fails to separate one dicarboxylic acid porphyrin from another, and many of the important porphyrins belong to this class. Since the carboxylic acid groups are separated from the resonating porphin system by aliphatic $-CH_2-CH_2$ groups, differences in substitution of the porphin ring by non-acidic side chains do not influence the pK of the acidic group and hence the partition. Recently, Chu, Green and Chu (1951) and Nicholas and Rimington have been able to separate meso- and proto-porphyrin esters by a paper chromatography with neutral solvents.

SUMMARY.

Oxidation of protoporphyrin dimethyl ester with permanganate in very dilute acetone solution gives a well crystallized equimolar mixture of chlorocruoroporphyrin and diformyldeuteroporphyrin esters with a 15 p.c. yield. This can be separated by alumina chromatography and the chlorocruoroporphyrin ester thus obtained has the same properties as chlorocruoroporphyrin ester from natural chlorocruorin.

No definite evidence could be obtained for the presence of an isochlorocruoroporphyrin in these preparations, but it appears likely that this was present in the impure preparations. No fraction could be obtained, however, which had the properties of the verd$_{NO_2}$ porphyrin ester of Alslev and Kiese, provisionally identified by these authors with isochlorocruoroporphyrin ester.

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SALMONELLA TYPES OCCURRING IN AUSTRALIA. 9. *S. ADELAIDE*.[1]

by NANCY ATKINSON, HEATHER G. GEYTENBEEK AND GWENDOLYN M. WOODROOFE.

(From Department of Bacteriology, University of Adelaide).

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S. adelaide was first described by Atkinson (1943) on the examination of one strain isolated from a fatal case of enteritis in Adelaide, South Australia. Since this first isolation numerous other strains of this type have been found in Australia and recorded in earlier papers of this series (Atkinson *et al.*, 1944, 1949, 1952). The present paper gives the characteristics of fifty strains of this type together with their sources. *S. adelaide* has now been found in rats and lizards, as well as in man. The majority of our strains came, however, from human cases of gastro-enteritis.

Cultural and biochemical reactions.

Fifty strains, including the original strain (No. 1) were investigated. All were Gram negative, motile bacilli growing freely on nutrient broth and agar. All were Stern glycerol positive, produced H_2S, and failed to form indole. In peptone water all strains rapidly formed acid and gas from glucose, maltose, mannitol, arabinose, xylose and rhamnose, and failed to attack lactose, sucrose, and inositol. Most of the strains were tested in trehalose from which they rapidly produced acid and gas, and in salicin which they failed to attack. One strain (No. 23) failed to ferment dulcitol in 14 days but all other strains readily produced acid and gas. The dulcitol-negative strain produced dulcitol-positive variants. No strain liquefied gelatine in 30 days at 22° C. In Jordan's tartrate all cultures rapidly became acid.

Serological identification.

Strains 2, 3, and 4 were identified by their ability completely to absorb the H and O agglutinins from *S. adelaide* antiserum. With subsequent strains, however, agglutinin absorption tests were not considered necessary. A culture was identified as *S. adelaide* by its reaction with representative H- and O-antisera. In slide tests all strains of *S. adelaide* were rapidly agglutinated by *S. adelaide* O-antiserum, which was markedly specific for this type. In tube tests, H suspensions of all strains of *S. adelaide* agglutinated readily in H antisera prepared against any Salmonella with the factor g. With absorbed H antisera specific for the factors f, p, q, m, u, s, t, agglutination occurred only with f serum.

Distribution and pathogenicity.

The sources of our strains of *S. adelaide* are given in Table 1.

[1] This work was partly supported by a grant from the National Health and Medical Research Council, Canberra.

TABLE 1.

Sources of 50 strains of *S. adelaide*.

Strain No.	Where and when isolated	Source		Brief Case History
		Patient, or animal	Specimen	
1	South Australia, 1943	adult, male	faeces, liver, spleen	enteritis, patient died.
2	South Australia, Jan., 1944	infant, aged 5 months	faeces	diarrhoea and vomiting. *S. adelaide* detected in faeces on six occasions during the first 14 days after onset; patient recovered.
3	South Australia, Dec., 1946	infant, aged 12 months	faeces	bilateral otitis media, vomiting and diarrhoea, four weeks after admission to hospital the serum agglutinated *S. adelaide* H suspension to a titre of 1/4000, patient died.
4	South Australia, July, 1946	infant, aged 3 months	faeces	vomiting but no diarrhoea, *S. adelaide* detected in faeces 13 days after onset, patient recovered.
5	South Australia, July, 1946	infant, aged 2 months	faeces	abdominal distention and blood in stools, *S. adelaide* detected in faeces on numerous occasions during seven weeks in hospital, patient recovered and was discharged while still carrying the Salmonella.
6	New South Wales, Jan. 1949	infant aged 9 months	faeces	gastro-enteritis.
7	New South Wales, Jan.,1949	infant, aged 16 months	faeces	bouts of diarrhoea and vomiting for nine months. Recovered after 1 month in hospital.
8	Queensland, June, 1947	infant, aged 9 months	faeces	gastro-enteritis, patient recovered.
9	Queensland, Oct. 1947	infant, aged 3 months	faeces	healthy carrier.
10	Queensland, Oct., 1947	child, aged 5 years	faeces	healthy carrier.
12	Queensland, Oct., 1947	adult, female	faeces	healthy carrier.
13	Queensland, Nov., 1947	infant, aged 9 months	faeces	dangerously ill with gastro-enteritis, recovered.
14	Queensland, Nov., 1947	infant, aged 8 months	faeces	gastro-enteritis, *S. boris-morbificans* also isolated later.
15	Queensland, Nov., 1947	child, aged 2 years	faeces	gastro-enteritis, *S. boris-morbificans* also isolated later.
16	Queensland, Nov., 1947	child, aged 5 years	faeces	acute dysentery, Flexner dysentery bacilli also isolated.
17	Queensland, Dec., 1947	infant, aged 10 months	faeces	gastro-enteritis, *S. boris-morbificans* isolated at first.
18	Queensland, Dec., 1947	infant, aged 9 months	faeces	gastro-enteritis
19	Queensland, Nov., 1947	adult, female	faeces	gastro-enteritis, *S. typhi-murium* also isolated.
20	Queensland, Nov., 1947	infant, aged 7 months	faeces	gastro-enteritis.
21	Queensland, Oct., 1947	infant, aged 6 months	faeces	healthy carrier, *S. adelaide* was isolated again 16 days after first isolation.
23	Queensland, Oct., 1947	rat	spleen and intestine	healthy rat caught in a Brisbane suburb.
25	Queensland, Oct., 1947	infant, aged 3 months	faeces	healthy carrier.
26	Queensland, Dec., 1948	child	faeces	gastro-enteritis, *S. typhi-murium* isolated at first.
27	Queensland, Jan., 1949	adult, female	faeces	gastro-enteritis.
28	Queensland, Feb., 1949	infant	faeces	mild gastro-enteritis.
29	New South Wales, April, 1949	infant, aged 3 months	faeces	gastro-enteritis.
30	Queensland, May, 1949	rat	bladder	healthy rat caught in vicinity of premises of the patient yielding strain 26.
31	South Australia, March, 1949	infant, aged 11 months	faeces	healthy carrier.
32	South Australia, June, 1949	infant, aged 10 months	faeces	mild diarrhoea.
33	New South Wales, Sept., 1949	infant, aged 6 months	faeces	mild gastro-enteritis, carrier state persisted for at least three months.
34	New South Wales, Sept., 1949	infant, aged 7 months	faeces	severe gastro-enteritis.
35	New South Wales, Sept., 1949	infant, aged 5 months	faeces	healthy carrier.
36	New South Wales, Sept., 1949	adult, female	faeces	healthy carrier.

TABLE 1 (continued).

Sources of 50 strains of *S. adelaide*.

Strain No.	Where and when isolated	Source		Brief Case History
		Patient, or animal	Specimen	
37	Queensland, Aug., 1949	infant, aged 11 months	faeces	whooping cough and vomiting. Loose motions 6 days after entering hospital and *S. adelaide* isolated. Condition deteriorated and patient died.
38	New South Wales, Sept., 1949	infant, aged 7 months	faeces	eczema and gastro-enteritis.
39	Queensland, Dec., 1949	child, aged 3 years	faeces	dysentery, *Sh. sonnei* isolated at first.
40	Queensland, Dec., 1949	child, aged 3 years	faeces	dysentery, *Sh. sonnei* isolated at first.
41	Queensland, Jan., 1950	child, aged 7 years	faeces	gastro-enteritis.
42	Queensland, Jan., 1950	child, aged 8 years	faeces	dysentery, Flexner dysentery bacilli isolated at the same time.
43	Queensland, March, 1950	child, aged 4 years	faeces	gastro-enteritis.
44	Queensland, March, 1950	child, aged 4 years	faeces	gastro-enteritis.
45	Queensland, March, 1950	infant, aged 11 months	faeces	gastro-enteritis.
46	Queensland, March, 1950	infant, aged 16 months	faeces	gastro-enteritis.
47	New South Wales, March, 1950	infant, aged 3 months	faeces	gastro-enteritis.
48	New South Wales, April, 1950	infant, aged 15 months	faeces	gastro-enteritis.
49	New South Wales, April, 1950	infant, aged 15 months	faeces	mild gastro-enteritis.
50	Queensland, May, 1950	lizard	rectal swab	apparently healthy.
51	Queensland, May, 1950	lizard	rectal swab	apparently healthy.
52	Queensland, June, 1950	sewer rat	gut	apparently healthy.
53	Queensland, July, 1950	lizard	large intestine	died in captivity.

Of the 50 strains of *S. adelaide* recorded here 44 came from humans, of whom 8 were healthy carriers and the rest were sufferers from enteritis in which other Salmonellas or dysentery bacilli were sometimes associated. Three of these patients died. The remaining 6 strains came from 3 rats and 3 lizards. All the rats and two of the lizards were apparently healthy but one of the lizards died in captivity. One rat was caught near the premises of the patient yielding strain 26.

Pathogenicity for mice: Strains Nos. 8 and 31 were inoculated intraperitoneally into mice, most of which died with the largest dose of 500×10^6 organisms. Lower doses failed to kill most of the mice, many of which appeared normal but still carried the organisms in the liver and the spleen 14 days after inoculation.

DISCUSSION.

Although six strains of *S. adelaide* were recovered from rats and lizards, no other animal strains were found among several hundred Salmonellas isolated from a wide variety of animals and recorded in the earlier papers of this series. Apart from the one lizard which died, the rats and lizards harbouring *S. adelaide* seemed unaffected and could be regarded as healthy carriers. Experimentally this Salmonella was not highly virulent for mice. It seems therefore to be mainly a human type generally found in mild gastro-

enteritis or healthy carriers. Mackerras and Pask (1949) followed up four of the healthy carriers (Nos. 9, 10, 11 and 21) and found that they carried the organism for weeks. Also a number of the gastro-enteritis cases became carriers for several weeks after recovery. This persistence of the organism after recovery was also noted by Dr. Stephen Williams (personal communication), who found that one mild case (No. 33) remained a carrier for at least three and a half months. This case, which was the original admission to the hospital ward, apparently gave rise to two symptomless carriers, a nurse and an infant (Nos. 35 and 36), and one severe case of gastro-enteritis (No. 34).

Although *S. adelaide* infection appears often to be asymptomatic or mild, one case (strain No. 13) was dangerously ill, and three cases (strains Nos. 1, 3, and 37) died. Strain No. 1 came from a patient who died of enteritis for which *S. adelaide* was the only detectable cause (Atkinson, 1943). The patients who provided strains Nos. 3 and 37 also died, but *S. adelaide* was not clearly the cause of death. One of these patients (No. 3) was admitted to hospital severely ill with bilateral otitis media and the Salmonella was not isolated until 11 days after admission. Nevertheless, vomiting and diarrhoea were prominent features of the latter part of her illness, and the antibody response (titre 1/4,000 for *S. adelaide* H suspension) suggested active infection. The other patient (No. 37) was admitted to hospital with whooping-cough and developed diarrhoea six days later. No other known cases of Salmonella infection were present in the ward at that time. Salmonella enteritis was listed among the causes of death together with pertussis, pneumonia, mastoiditis, and multiple haemorrhages.

In its effect upon humans *S. adelaide* thus resembles most of the common Salmonellas in producing mild to severe gastro-enteritis but differs somewhat from the other Salmonellas in its frequent occurrence in healthy individuals, and in producing a rather persistent carrier state in recovered cases.

SUMMARY.

The characteristics and sources of fifty strains of *S. adelaide*, of which only one strain had previously been adequately described, are given. Forty-four strains came from humans of whom eight were healthy carriers, and 36 were cases of gastro-enteritis. The cases were mainly mild and often became carriers for many weeks.

Three strains were obtained from healthy city rats and three strains came from lizards.

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QUANTITATIVE STUDIES ON SOME WATER-SOLUBLE ORGANIC CONSTITUENTS ASSOCIATED WITH VERTEBRATE KERATIN

by A. BOLLIGER AND R. GROSS[1]

(From the Gordon Craig Research Laboratory, Department of Surgery, University of Sydney).

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In previous communications (Bolliger, 1949, 1951; Bolliger and Gross, 1951), it has been stated that the extradermal fur and hair of mammals contain varying amounts of water-soluble organic non-protein substances and certain inorganic compounds in addition to the insoluble keratin. Most of these observations were made on rabbit fur and the question arose whether similar deposition occurs generally in vertebrate keratinous integument and its appendages. To answer this question, the hair of 10 different species of mammals, the feathers of 5 species of birds and the shed keratinous skin of 11 species of snakes have been examined for uric acid, pentose, substances reducing before and after hydrolysis, and phenols. Determinations of desoxyribose were also carried out in many instances and guanosine was isolated from rabbit fur.

METHODS.

The preparation of aqueous extracts of keratinous material.

Clipped fur, plucked feathers and cut-up shed snake skin were boiled for about 30 minutes in a conical flask, containing sufficient water to prevent serious bumping. The aqueous extract was decanted and filtered and as much liquid as possible removed. This process was usually repeated five times or until no more colour reaction for uric acid or phenol could be obtained from the extract. The combined extracts were then concentrated to a convenient volume either over a flame or on a water bath *in vacuo*.

Uric acid.

Due to the high phenol content of certain types of keratinous material, the direct application of the uric acid reagent of Folin (1933) to the aqueous extracts frequently leads to results which are definitely too high. In the "indirect" method of Folin, uric acid is isolated in the form of its silver salt prior to its determination (1934). Both methods, direct and indirect, were used in our investigation. Folin's "indirect" method originally described for the determination of uric acid in urine was adapted for the aqueous extracts of keratinous material as follows:

[1] Working under a grant from the National Health and Medical Research Council.

To 5 ml. of the aqueous extract in a centrifuge tube 0·5 ml. of a solution containing 1 p.c. sodium chloride and 2 p.c. sodium acetate in 0·01 p.c. acetic acid were added. Then after the addition of 3 ml. of a 5 p.c. silver nitrate solution the mixture was centrifuged for 4 to 5 minutes. After decanting and draining the clear supernatant solution, the sediment was dissolved in 10 ml. of the urea cyanide solution (Folin, 1933). To the resulting uric acid solution. 5 ml. of water and 4 ml. of Folin's uric acid reagent were added. After standing for 20 minutes, the volume was made up to 25 ml. and the resulting blue colour was compared with a standard approximately the same depth of colour.

Total phenols.

Uric acid was removed from the aqueous extract as the insoluble silver urate (Volterra, 1942). Then Folin and Ciocalteu's reagent (1927) was added to an aliquot of the extract and the blue colour produced compared with that of a standard prepared from phenol. The results obtained are referred to as total phenols present in the keratinous material.

Directly reducing substances.

These were estimated as glucose by the method of Somogyi (1926) which was applied directly to the suitably concentrated extract.

Substances which become reducing after hydrolysis.

One volume of the suitably concentrated aqueous extract of keratinous material was hydrolyzed with approximately 1/20th volume of concentrated hydrochloric acid by heating in a boiling water bath for one hour. After neutralizing the acid with solid sodium bicarbonate the total reducing substances present were determined as glucose by the method of Somogyi (1926). From these total reducing substances the directly reducing substances were subtracted. The difference represents the substances which become reducing after hydrolysis expressed as glucose.

Pentose.

The pentose content of the aqueous extract of keratinous material was determined by the orcinol method as described by McRary and Slattery (1945).

In additon. Tauber's qualitative reagent (1937) consisting of benzidine and glacial acetic acid was employed, in order to distinguish between the free pentoses and nucleotides on the one hand and nucleosides and pentosans on the other hand, the latter two requiring hydrolysis with mineral acid to give a positive reaction.

For example, 0·2 ml. of concentrated extract were boiled vigorously with 1·0 ml. of a 4 p.c. solution of benzidine in glacial acid (Tauber's reagent). After cooling 0·5 to 1·0 ml. of water were added. The presence of pentose is indicated by the appearance of a pink to red colour. If the resulting colour was doubtful owing to impurities, it was extracted with 1 ml. of amyl alcohol. Sufficient water was added to ensure separation of the layers.

For hydrolysis 0·1 ml. 5N HCl was added to 1·0 ml. of extract which was then heated in a water bath for 30 minutes simultaneously reducing the volume to about 0·2 ml. After this 1·0 ml. of Tauber's reagent was added, the mixture boiled and 1·0 ml. of water added after cooling.

Desoxyribose.

This determination was carried out by a modification of Dische's diphenylamine reaction as described by Davidson and Waymouth (1944). Desoxyribose does not give a positive Tauber test.

RESULTS.

Keratinous snake skin.

As shown in Table 1, the aqueous extract of all keratinous snake skins examined contains appreciable amounts of uric acid, total phenols, directly reducing substances, substances which become reducing after hydrolysis, pentose, and desoxyribose. Quantitatively there is a considerable variation among different species of snakes for all the six substances determined.

TABLE 1.

Findings on snake skins.

Type of snake	Uric acid "Direct" Mg. p.c.	Uric acid "Indirect" Mg. p.c.	Phenols Mg. p.c.	Reducing substances "Direct" Mg. p.c.	Increase after Hydrolysis Mg. p.c	Pentose Mg. p.c.	Desoxyribose Mg. p.c.
Green Python	79	71	63	232	116	141	
African Python	124	113	119	234	65	127	40
Death Adder	281	248	218	470	175	159	70
Copper Head	290	263	110	407	70	187	64
Brown	299	282	210	330	242	172	68
Japanese Tree	165	137	216	668	400	346	71
Tiger	315	222	207	506	1,319	544	80
Black	537	250	172	383	137	181	60
Black*—dorsal	169	141	168	435	138	252	130
Black*—ventral	120	111	121	213	207	150	190
Diamond—dorsal	226	188	82	335	136	203	58
Diamond—ventral	196	125	103	227	125	100	24
Boa—dorsal	111	90	91	205	104	130	46
Boa—ventral	235	103	52	168	74	74	38

* Skin obtained by skinning.

Pentose (92 to 544 mg. p.c.) and desoxyribose (40 to 190 mg. p.c.) form a varying fraction of the reducing substances but in all cases, with the exception of the tiger snake, the sum of pentose and desoxyribose exceeds the amount of substances which become reducing after hydrolysis. Some of the pentose may therefore be expected to be present in the free state. This was confirmed by a positive Tauber test without prior hydrolysis. In this connection it may be mentioned that the precipitate obtained by adding 9 volumes of alcohol to aqueous snake skin extracts does not give a positive iodine test for glycogen.

Total phenol values were found to vary from 52 to 218 mg. p.c. (average 139 mg. p.c.) and uric acid, determined by the indirect method, from 71 to 282 mg. p.c. (average 167 mg. p.c.).

In two specimens of black snake the morphologically different dorsal and ventral skins were examined separately. With the exception of desoxyribose which was found to be higher in the ventral skin, the dorsal skin extract showed

significantly larger amounts of the substances examined. One of these skins was obtained by removing it from a black snake before it had actually been shed.

Directly reducing substances appear to be high (average 344 mg. p.c.) compared with findings on birds (average 157 mg. p.c.). The same applies to substances reducing after hydrolysis, which in the tiger snake reach more than 1,300 mg. p.c. The value found for pentose in this snake is also exceptionally high, i.e. 544 mg. p.c.

In all snakes examined, the combined amounts of pentose and desoxyribose found only account for a fraction of the total reducing substances. The nature of the remaining reducing substances is as yet unknown.

Birds.

The analytical findings for birds' feathers (Table 2) were similar to those in snake skin. All the six substances previously mentioned were present, though in amounts usually smaller than observed in the keratinous integument of snakes.

TABLE 2.

Findings on birds' feathers.

	Uric acid			Reducing substances			
	"Direct"	"Indirect"	Phenols	"Direct"	Increase after hydrolysis	Pentose	Desoxyribose
	Mg. p.c.	Mg. p.c.	Mg. p.c.	Mg. p.c.	Mg. p.c.	Mg. p.c.	Mg. p.c.
Dove	187	145	60	226	22	213	41
Fowl—white	123	115	84	132	54	57	77
Duck	50	48	67	139	28	46	—
Wild Duck, barbs	54	59	117	211	78	161	—
Wild Duck, shafts	41	31	79	125	54	74	—
Wild Duck, barrel	44	26	54	64	24	25	—
Pigeon, barbs	199	188	89	235	39	198	55
Pigeon, shaft	48	29	73	148	21	97	32
Pigeon, barrel	31	16	52	148	25	67	33

The amount of substances which become reducing after hydrolysis was frequently small and particularly in the dove amounted to only 22 mg. p.c. expressed as glucose, while the pentose was 213 mg. p.c. Pentoses were therefore expected to be found amongst the directly reducing substances. This conjecture was supported by a positive Tauber test on the unhydrolyzed extract. These pentoses may be present as free pentose or nucleotides. The sum of pentose and desoxyribose practically equals the total reducing substances of dove feathers and pigeon barbs.

Highly concentrated birds' feather extracts give a blue colour with iodine solution.

In the wild duck and the pigeon, the larger feathers were separated into barbs, shafts and barrels. The water soluble components were found to be highest in the barbs and lowest in the barrel (which is directly connected with the skin). The values obtained for the shafts were frequently somewhat higher than those obtained from the barrels. The difference between "direct" and "indirect" uric acid is less in barbs than in shafts and barrels.

Mammals.

Determinations of the six substances were made both on marsupials and eutheria. As shown in Table 3, there are wide variations among different mammals even if man, whose hair is characterized by an exceptionally low content of water—extractable substances, is excluded from consideration. For example, the substances which become reducing after hydrolysis reach comparatively high figures in some species of kangaroo, and particularly in the rabbit (400 mg. p.c. and more). Values of less than 70 mg. p.c. were obtained in all other mammals examined, while in human hair the values were practically nil. In many instances, as mainly with birds, the amounts of substances reducing after hydrolysis were much less than those of the corresponding pentose. In agreement with this a positive Tauber test was obtained on the unhydrolyzed extracts of these furs, suggesting the presence of free pentose or nucleotide.

TABLE 3.

Findings on mammalian fur and hair.

	Uric acid		Reducing substances				
	"Direct"	"Indirect"	Phenols	"Direct"	Increase after hydrolysis	Pentose	Desoxyribose
	Mg. p.c.	Mg. p.c.	Mg. p.c.	Mg. p.c.	Mg. p.c.	Mg. p.c.	Mg. p.c.
Black gloved wallaby	160	163	170	288	122	133	—
Swamp wallaby	165	143	130	368	157	162	—
Red kangaroo	350	191	120	504	238	160	—
Ringtail 'possum	145	99	210	325	30	145	—
Phalanger, dorsum	70	40	220	237	64	145	80
abdomen	230	100	640	492	33	159	160
Rabbit	570	290	60	201	449	160	50
Guinea-pig	93	73	63	194	31	98	40
Sheep†							
Lincoln	21	22	32	234	58	88	
Corriedale	34	40	44	175	35	80	
Dog	221	133	51	146	32	81	73
Human hair‡	9	6	28	63	4	30	20

† Previously extracted with ether. ‡ Male—age 53.

In the fur of the rabbit (Bolliger and McDonald, 1948) and of the kangaroo, an amorphous substance was isolated which has proved to be mainly glycogen and which gives a typical glycogen reaction with iodine.

The uric acid content of the animals (indirect method) varied from 22 to 290 mg. p.c. In man it was only 6 m.g. p.c.

The marsupial, *Trichosurus vulpecula*, requires special mention because most analytical values obtained for ventral fur are markedly higher than those obtained for dorsal fur. This phenomenon is particularly marked with regard to the total phenol content, which reaches as much as 1,000 mg. p.c. in hairs from the abdomen. The fur of all herbivorous marsupials examined so far generally exhibits a higher phenol content than that of eutheria (Bolliger and Gross, 1951).

Guanosine.

In addition to the quantitative estimations on skin, feathers and furs, attempts were also made to isolate from the aqueous extracts hitherto unrecognized compounds contained in it. For example, 1,000 gm. of rabbit fur were extracted with boiling water and the extract evaporated to dryness. The residue was then extracted with alcohol for several hours in the Soxhlet apparatus. The alcohol extract yielded about 20 mg. of white crystals.

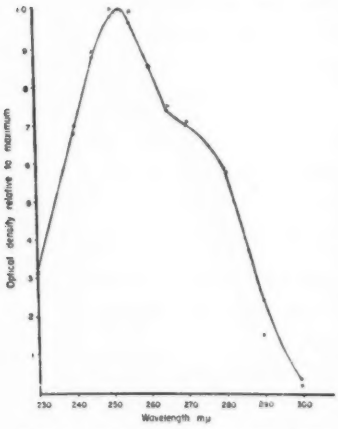

Fig. 1. Relative absorption curve of guanosine isolated from rabbit fur. 1·0 = maximum absorption. x = guanosine as determined by Hotchkiss (1948). His observations were made only at intervals of 5 or 10 mμ.

On re-extracting the residue with hot water and concentrating the extract to 100 ml., a brown substance was obtained on cooling. This substance was suspended in water and dialyzed through cellophane. On evaporation the dialysate yielded crystals which on repeated recrystallization from water became white needles decomposing at 230° C. The total yield was 0·07 gm. from 100 gm. of rabbit fur. This compound was examined chemically, spectroscopically and by partition chromatography. All these findings proved that the needles are the riboside of guanine, guanosine. Qualitatively it was found to consist of guanine and pentose, the pentose content of the water-free material being 54 p.c. (theoretical value 53 p.c.) (Bolliger and Gross, 1951 b, preliminary communication). It gave a positive Tauber's test after previous hydrolysis with mineral acid. Ultra violet absorption data were similar to those recently obtained by Hotchkiss (1948) for guanine riboside (Fig. 1). As with

the guanosine described in the literature (Levene, 1920), our compound crystallized from water with two molecules of water.

Analysis: C, 37·6; H, 5·5; N, 22·0.
Calcd. for: $C_{10}H_{13}O_5N_5 \cdot 2H_2O$ C, 37·6; H, 5·3; N, 21·9.

The crystals obtained by alcohol extraction were found to be identical with the compound obtained by dialysis. Prolonged alcohol extraction may be a more convenient method of isolating guanosine.

DISCUSSION.

In the present investigation Folin's "indirect" method of uric acid determination was used in conjunction with the direct method employed previously (Bolliger and Hardy, 1945), primarily to avoid interference by any high content of phenolic substances. This "indirect" method usually gives lower figures than the "direct" method (Tables 1, 2 and 3), but these lower figures probably approximate more closely to the true values. In the majority of determinations, the difference between the two methods amounted to only 5–20 p.c., but in certain instances, e.g. the rabbit, the "indirect" values are very much lower. Only about 60–80 p.c. of uric acid added to aqueous rabbit fur extract could be recovered by this method; moreover, gravimetric determinations also yielded values from 20–35 p.c. higher than those obtained by the "indirect" colorimetric method which is consistent with the assumption that the "indirect" values are too low in the case of the rabbit. It therefore appears that the silver salt precipitation of uric acid may occasionally remain incomplete. In general, no method of uric acid determination was completely satisfactory when applied to the complex mixture of compounds met with in some of the extracts of keratinous tissue. The "direct" method of Folin furnishes maximum, the "indirect" minimum values, but in the majority of cases the results of the two methods are near enough to be of significance.

The uric acid content of all keratinous tissues examined is high if compared with the uric acid content of other tissues and equally high values were obtained in uricotelic birds and snakes and ureotelic mammals.

In the keratinous tissues examined, substances which became reducing after hydrolysis varied from 4 to 1,319 mg. p.c. In mammals such as the rabbit (Bolliger and McDonald, 1948), and kangaroo where these hydrolyzable compounds are comparatively plentiful, they were considered wholly as glycogen. This view must be modified to a certain extent, since the riboside of guanine (guanosine) found to be present in rabbit fur extract would also become reducing after hydrolysis in addition to the glucose formed from glycogen, and would account for approximately 10 p.c. of the hydrolyzable reducing substances. In some extracts of birds' feathers, the combined pentose and

desoxyribose values closely approach that of total reducing substances. Here the total reducing substances are obviously represented mainly or in some instances even solely by pentose and desoxyribose.

In this connection it may be pointed out that although Dische's diphenylamine reaction is given by all desoxysugars, in the present circumstances the desoxysugars are tentatively interpreted as desoxyribose. The substance referred to as pentose in all probability consists mainly of ribose as indicated by the presence of the riboside of guanine in the extract of rabbit fur.

The reaction for total phenols, though not very specific, gives a useful indication of the relative phenolic content of keratinous substances from different species. It must be emphasized, however, that none of these phenolic substances has been identified except phenol itself, which occurs in only comparatively small amounts. The exceptionally high phenol content of the fur of phalangers and other marsupials may have originated from their diet of leaves which contain large amounts of phenolic substances (Bolliger and Gross, 1951, a).

Although the quantities of the six substances determined vary considerably in different species, all these compounds are constantly present. We are therefore not dealing with an isolated phenomenon but with a series of compounds common to keratinous integument in general. The analytical findings on snakes are of special interest inasmuch as they were obtained from *stratum corneum* and not from keratinous appendages as with birds and mammals.

The formation and presence of these compounds in association with the keratin may be explained as follows:

In the process of keratinization, a water insoluble protein, keratin, is synthetized. This protein is accompanied by comparatively simple water soluble products of the catabolism occurring in conjunction with the protein synthesis.

In the present communication, uric acid, substances reducing before and after hydrolysis, ribose, desoxyribose and phenol were investigated. Reference is made to the isolation of guanosine. Most of these substances are related to ribonucleic and desoxyribonucleic acid. These nucleic acids, present in the epidermal tissue, disappear in the process of keratinization and it is assumed that the purines contained in them undergo the classical catabolism terminating in uric acid. Together with other products of nucleic acid degradation such as ribose, desoxyribose, guanosine and some substances as yet undetermined, uric acid is left residing with the keratinous structures formed. This process occurs in all vertebrates independent of the typical metabolic pattern of their species.

SUMMARY.

The aqueous extract of keratinous structures such as fur of mammals, feathers of birds and shed snake skin were analysed for uric acid, substances reducing before and after hydrolysis, pentose (probably ribose), desoxyribose, and phenols. These six constituents were found to be present in varying amounts in all the keratinous tissues examined. Frequently they occurred in physiologically large quantities of several hundred milligrams per cent.

Substances reducing before and after hydrolysis could not always completely be accounted for by pentose and desoxyribose. There is evidence that the pentose is present as combined pentose of the nucleoside type, as proven by the isolation of guanosine, or as free pentose or nucleotides.

Combined hexoses in the form of glycogen were isolated from the aqueous extract of rabbit and kangaroo fur.

Phenolic substances were particularly plentiful in herbivorous marsupials.

The occurrence of large amounts of uric acid in keratinous tissue has been confirmed by using Folin's "indirect" method in addition to techniques used previously.

Pentose, desoxyribose and uric acid are considered to have been derived from nucleic acid present in the epidermis. In the process of keratinization they have become deposited within the structures formed. This is supported by the isolation from rabbit fur of guanosine, an intermediary of nucleic acid catabolism.

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HYDROGEN PEROXIDE ACCUMULATION DURING GROWTH OF THE *PNEUMOCOCCUS*

by D. I. ANNEAR AND D. C. DORMAN

(From the Institute of Epidemiology and Preventive Medicine, the Prince Henry Hospital, Sydney).

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Since the early work of McLeod and Gordon (1922) on the occurrence of peroxide in cultures of the pneumococcus and certain other bacteria, numerous other workers have contributed observations to this and allied fields and have confirmed the results of the work cited.

Molland (1947), however, using polarographic methods, was unable to detect H_2O_2 in pneumococcal and other bacterial cultures and threw doubt on past observations in this field.

Aussen, Kingma Boltjes and Wöstmann (1950) criticized Molland's methods and published polarograms showing evidence of appreciable H_2O_2 production by the pneumococcus.

It is indeed difficult to disbelieve, in the face of available evidence, that bacteria do produce, under certain cultural conditions, considerable amounts of H_2O_2. Perhaps the best evidence for bacterial peroxide production is the observation of McLeod and Gordon that the addition of catalase to well aerated and turbid cultures of the pneumococcus resulted in abundant evolution of oxygen.

Progress in this field has long been impeded by the lack of application of satisfactory methods for quantitative determination of H_2O_2 in bacterial cultures. Apart from the specialized techniques of polarography, the methods which have been most commonly employed are those which depend upon peroxidase activity (e.g. Main and Shinn, 1939). These methods have undoubtedly been useful, but are unsatisfactory for quantitative work because of the instability of the reagents employed and because of the relatively rapid fading of the produced blue colour upon which the measurements are based.

A colorimetric method which has neither of these disadvantages and which is simple to perform, relatively sensitive and apparently specific, is the titanium method (Allsopp, 1941). Although it has been used by various workers in a number of fields (Eisenberg, 1943; Bonet-Maury, 1944; Wyss *et al.*, 1947; Humpoletz, 1949) it does not appear to have been applied to the measurement of bacterial peroxide production. The experiments recorded here contain a report of the application of the titanium method in the measurement of H_2O_2 produced during the growth of the pneumococcus.

The autolysis that occurs in pneumococcal cultures is a well known phenomenon, though its relationship to other phenomena of cell activity such as the viable population and H_2O_2 accumulation does not appear to have been studied.

In the present work an attempt has been made to present some information on the relationships between oxygen supply, the viable population, turbidity and H_2O_2 accumulation in cultures of the pneumococcus.

The pneumococcus (P1) employed was a strain isolated from a throat culture.

METHODS.

Media. The nutrient broth and fluid used to dilute the pneumococcal suspensions for counting purposes were of the same composition as described by Annear and Hayes (1950). Viable counts were made on a blood agar medium (Ward and Rudd, 1938).

Viable counts. The method of Miles and Misra (1938) was used.

H_2O_2 measurement.

The titanium reagent was 0·05 M titanium in 4 N H_2SO_4 (Humpoletz, 1949) and was prepared by adding titanic chloride (B.D.H.) to the H_2SO_4.

A Klett Summerson photometer with "KS 42" filter and 12 mm. tubes was used for the colour measurements.

It was found that it was unnecessary to remove the cells from the pneumococcal cultures as supernatant fluids and the turbid suspensions from which they were derived gave identical results with respect to colour development. The colour, however, tended to fade more rapidly in the presence of the cells, but was quite stable for at least 30 minutes after development.

An essential condition of the test is the final pH of the preparation, which, though by no means critical, must be low enough to prevent precipitation of the Ti and high enough to prevent acid precipitable material separating in the broth.

The following conditions were found to be satisfactory for the colour measurement:

Test: 2 ml. culture + 2 ml. of 4 N H_2SO_4 + 2 ml. Ti Reagent.
Blank: 2 ml. culture + 4 ml. of 4 N H_2SO_4.

Where necessary the pneumococcal cultures were diluted in distilled H_2O to ensure convenient scale readings.

Calibration with commercial H_2O_2 under the conditions described revealed that the lowest concentration of H_2O_2 that could be measured with reasonable accuracy was of the order of 3 μg./ml.

EXPERIMENTAL.

Fig. 1 shows the conditions and results of an experiment in which the viable populations and H_2O_2 production of three broth cultures of the pneumococcus have been recorded.

The culture flasks, plugged with cotton wool, were incubated in a chamber the atmosphere of which was ordinary laboratory air. In the case of culture C, oxygen was bubbled through the broth continuously throughout the experiment. Cultures A and B were not actively aerated in any way, but depended for their oxygen upon natural diffusion from the atmosphere. The disturbance to which

the cultures were subjected during sampling operations undoubtedly resulted in some additional aeration. While no accurate assessment of the difference was made, it can be assumed that, because of the different shapes of the vessels, culture B was subjected to more aeration than culture A.

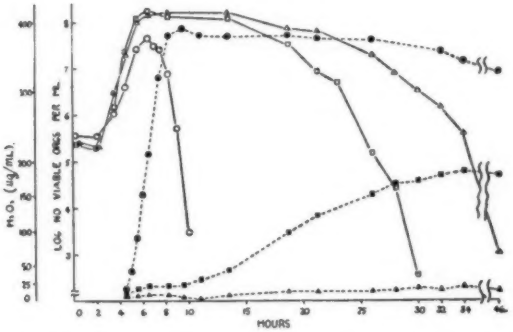

Fig 1. Growth of *Streptococcus pneumoniae* in nutrient broth at 37° C.

	Viable count	H_2O_2
A. Growing in 800 ml. broth in 1 l. Erlenmeyer flask	△——△	▲- - -▲
B. Growing in 800 ml. broth in 3 l. Erlenmeyer flask	□——□	■- - -■
C. Growing in 800 ml. broth in 2 l. Erlenmeyer flask (oxygenated)	o——o	●- - -●

It may be seen from the Figure that the general effect of aeration was to shorten the life of the cultures and to increase the accumulation of H_2O_2. Some marked differences in the times of maximal peroxide accumulation are also apparent. The H_2O_2 level in culture C rose maximally during the logarithmic growth phase and continued to rise though not so rapidly toward the end of this phase and during the decline phase. In the case of cultures A and B, the H_2O_2 levels rose during the logarithmic growth phase, became stationary (or fell in the case of A) during the earlier part of the stationary growth phase and rose maximally during the decline phase of growth.

The main observations that were made with respect to the changing turbidities of the cultures were as follows:

(1) At that time (approximately the same for all three cultures) when the viable populations reached their maxima, the turbidity of culture C was as high, if not higher, than that of A or B.

(2) Culture C was sterile before any appreciable fall in turbidity was observed, whereas with cultures A and B, the decline in turbidity accompanied the decline of the viable population.

DISCUSSION.

Earlier work in this field was based upon methods which permitted detection but not accurate measurement of H_2O_2 in bacterial cultures. The findings presented here do not add very much to previous knowledge, but the methods used have enabled the information to be presented on a quantitative basis.

The H_2O_2 level present at any time in pneumococcal cultures must represent the balance between that produced and that destroyed. In ignorance of the many factors which are operative in these processes, it is premature to attempt to explain the various effects observed. That peroxide accumulation is related to oxygen tension, however, seems firmly established.

Avery and Morgan (1924) noticed that the more cultures were deprived of air, the longer was the lag before H_2O_2 appeared. In the present work it has been shown that H_2O_2 may be formed during the early stages of growth in poorly aerated cultures though in concentrations which might easily escape detection. However, it appears to be true that maximal accumulation of H_2O_2 in such cultures occurs during the later stages of growth when the viable population is falling and autolysis is occurring. The demonstrations by Avery and Neill (1924a, b) that H_2O_2 could be produced by "resting" pneumococcal cells and also in the absence of living cells may aid in the explanation of this finding.

It is generally believed that the rapid onset and rate of the decline phase in aerated pneumococcal cultures is attributable to peroxide accumulation and the evidence presented here certainly does not contradict that view. However, the point is by no means proved. It would, for example, be interesting to test the effect of vigorous oxygenation on a culture supplied with sufficient catalase to destroy the peroxide as rapidly as it was produced. Similarly, it would be of interest to measure the death rates in pneumococcal cultures treated with commercial peroxide in amounts comparable to those produced in the cultures.

It was interesting to observe that although the viable population of culture C did not reach that of A or B, its turbidity was as high as, if not higher than, that of either of the other two cultures. This finding suggests that in culture C, either the organisms were larger or that they were able to divide but not to survive in the oxygenated medium with its attendant high peroxide content.

The observations made suggest that aeration and the peroxide accumulation that occurs with it, have little if any effect on the autolysis of the cultures, but accurate data to support this belief are yet to be obtained.

SUMMARY.

The well known connection between peroxide accumulation and the rapid decline phase which occurs in aerated cultures of the pneumococcus was demonstrated by quantitative measurements.

Some observations were also made on the changes in turbidity associated with the growth and autolysis of the organism.

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